Body size, reproduction and size-selective harvesting: an experimental study in zebrafish (*Danio rerio*)

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LIST OF PAPERS

This thesis is based on the five papers, which are referred to in the text by their roman numbers (I – V). Papers I, II, IV and V are reprinted with permission of the publishers.

Paper I

Uusi-Heikkilä, S., Kuparinen, A., Wolter, C., Meinelt, T., O'Toole, A., and Arlinghaus, R. (2011)

Experimental assessment of probabilistic maturation reaction norm: condition matters.

Proceedings of the Royal Society B **278**: 709-717

Paper II

Uusi-Heikkilä, S., Kuparinen, A., Wolter, C., Meinelt, T., and Arlinghaus, R. (2012)

Paternal body size affects reproductive success in laboratory-held zebrafish (*Danio rerio*).

Environmental Biology of Fishes **93**: 461-474.

Paper III

Uusi-Heikkilä, S., Böckenhoff, L., Wolter, C., and Arlinghaus, R.

Differential allocation in zebrafish is robust against the previous exposure to different-quality males.

Submitted

Paper IV

Uusi-Heikkilä, S., Wolter, C., Meinelt, T., and Arlinghaus, R. (2010)

Size-dependent reproductive success of wild zebrafish *Danio rerio* in the laboratory.

Journal of Fish Biology **77**: 552-569.

Paper V

Uusi-Heikkilä, S., Wolter, C., Klefoth, T., and Arlinghaus, R. (2008)

A behavioral perspective on fishing-induced evolution.

Trends in Ecology and Evolution **23**: 419-421.

CONTENTS

ABSTRACT (ENGLISH)

Body size is a fundamentally important trait for fitness in many animal species because it correlates positively with survival and reproductive success. In many fish species, large females exhibit higher fecundity and produce higher quality offspring compared to small females. Similarly, male body size can affect offspring quality and early life-history traits but the importance of these effects to the reproductive biology of fish is poorly studied. The extent to which variation in reproductive success is explained by parental body size is an important research topic because size-selective fishing usually reduces the average size of reproducing adults in a population. In my dissertation, I studied the parental size effects on reproductive success in a model species (zebrafish, *Danio rerio*). I also studied the effects of size-selective harvesting on body size, maturation and reproductive output. Body size and condition factor were important determinants of the initiation of maturation in zebrafish. Large females were found to have higher reproductive success compared to small females and a significant effect of male body size on early life-history traits was documented. I found that large males were also favored by the females resulting to differential allocation of reproductive resources toward large males. The maternal- and paternal-size effects ultimately led to elevated reproductive success of experimental spawning stocks consisting of large or random-sized individuals compared to spawning stocks consisting of small individuals. Size-selective harvesting induced rapid phenotypic and genetic changes, which persisted after selection was halted. This suggests that fishing-induced changes might be hard to reverse. My results emphasize the importance of body size to the reproductive biology of zebrafish and suggest that protecting large fish might be important to maintain the reproductive potential of exploited fish stocks.

Keywords: Body size, reproductive success, maturation, maternal effect, paternal effect, mate choice, differential allocation, size-selective harvesting, zebrafish (*Danio rerio*)

ABSTRACT (GERMAN)

Die Körpergröße ist von großer Bedeutung für die Fitness vieler Tiere, weil sie positiv mit Überleben und Reproduktionserfolg korreliert ist. Große Rogner vieler Fischarten sind fruchtbarer und produzieren Nachkommen von höherer Qualität verglichen mit kleineren Weibchen. Auch für Milchner einiger Fischarten wurde ein Einfluss der Körpergröße auf frühe Lebensstadien nachgewiesen. Der größenabhängige paternale Effekt verglichen mit maternalen Effekten ist weniger gut untersucht. Das Verständnis der Variation im Reproduktionserfolg als Funktion der Körpergröße der Laicher ist wichtig, weil die Fischerei die Durchschnittsgröße des Laicherbestands reduziert. In vorliegender Dissertation wurden in Laborversuchen an Zebrafischen (*Danio rerio*) größenabhängige paternale und maternale Effekte auf den Reproduktionserfolg und die Auswirkungen größenselektiver Entnahme auf Körperlänge, Reifung und Reproduktionserfolg untersucht. Die Köperlänge und Kondition waren wichtige Determinanten der Reifung bei Zebrafischen. Größere Rogner zeigten höheren Reproduktionserfolg als kleinere Fische und ein signifikanter Einfluss der Milchnerkörperlänge auf die frühen Lebensstadien ihrer Nachkommen wurde dokumentiert. Längere Männchen wurden von Rognern auch bei der Paarung bevorzugt. Die größenabhängigen maternalen und paternalen Effekte waren ausschlaggebend für den erhöhten Reproduktionserfolg von Zebrafischlaichbeständen, die, verglichen mit kleinen Laichern, aus großen oder zufällig zusammengesetzten Individuen zusammengesetzt waren. Die größenselektive Entnahme führte zu phänotypischen und genetischen Veränderungen, die nach Einstellung der experimentellen Befischung persistierten. Das deutet an, dass die durch die Fischerei ausgelöste Evolution schwierig umkehrbar sein könnte. Die Köpergröße ist von überragender Bedeutung in der Reproduktionsbiologie des Zebrafisches und der Schutz großer Laichfische kann wichtig für den Erhalt der Reproduktionskapazität von befischten Beständen sein.

Schlagwörter: Körpergrößen, Reproduktionserfolg, maternale Effekte, paternale Effekte, Reifung, Partnerwahl, differentielle Allokation, größenabhängige Entnahme, Zebrafisch, (*Danio rerio*)

1 BACKGROUND

Body size has long been recognized as a fundamentally important trait influencing several fitness-related aspects in an individual's life, such as survival and reproduction (Peters 1983). In fish, large body size is generally considered advantageous as it has been shown to correlate positively with fecundity (Wootton 1998), offspring quality (Heath 1998, Berkeley et al. 2004), mating success (Andersson 1994) and longevity (Roff 1992, Hutchings 1994) and negatively with reproductive costs, such as winter mortality (Lindstrom 1998, Schultz and Conover 1999). Furthermore, body size can be positively associated with the ability to provide parental care (Wiegmann and Baylis 1995), probability of attracting mates (Foote 1988) and ability to acquire and defend nest sites (Vandenberghe and Gross 1989). In fact, the link between body size and fitness is so well established that size is frequently used as a surrogate for fitness when direct measurements of lifetime fitness are impractical or impossible (Dmitriew 2011). Although large body size might be favored by natural selection, human harvest of animals in the wild often selects against large body size (Coltman et al. 2003, Fenberg and Roy 2008, Hutchings and Fraser 2008).

Fisheries selection typically operates on body size and most harvesting strategies increase adult mortality and reduce the relative abundance of large individuals in a population (e.g., Hutchings and Reynolds 2004, Kuparinen et al. 2009). By decreasing stock densities, fishing reduces intra-specific competition and this can result in greater per capita availability of resources (Rose 2001, Poysa et al. 2004). Growth in fish is often very plastic and responds to the current environmental conditions (Weatherley 1990), thus higher level of food resources may enable faster growth and earlier maturation at a potentially larger size (Stearns and Crandall 1984, Reznick 1990, Rochet 1998). However, the effects of fishing often go beyond simply releasing a stock from intra-specific competition. For example, the compensatory responses, related to accelerated growth and maturation, may further be affected by changes in the physical (e.g., temperature; Atkinson 1994, Kjesbu et al. 1996) or biotic environment (e.g., predation regime, Reznick and Endler 1982, Ball and Baker 1996). Furthermore, fisheries theory assumes that phenotypic variation is purely environmental and that removing biomass stimulates new production for further harvest without changing the intrinsic (i.e., genetic) characteristic of the selected traits (e.g., Christie and Regier 1988, Shuter et al. 1998). Thus, fisheries theory typically neglects the genetic influences on life-history traits and ignores the potential for fisheries-induced evolution (Conover et al. 2005).

High fishing mortality combined with size-selectivity may have negative effects on the demographic structure of the exploited stock. For example, reducing the number of spawning fish might be disruptive to population's reproductive success (the Allee effect) because a small number of spawners is potentially not able to produce enough offspring to regenerate the stock (Myers et al. 1995). More importantly, the spawning stock of an exploited population may not only consist of few individuals but also of small individuals due to the size-selective fishing practices (Myers et al. 1997, Rose et al. 2000). The positive relationship between female body size and fecundity is well-established (Wootton 1998) and maternal-size effects on other reproductive traits have also been documented in many marine and freshwater fish species (Green 2008, Marshall et al. 2008). Thus, truncating the size distribution by selectively removing large females has been suggested to have a strong influence on population dynamics, including fisheries yield (Law and Grey 1989, Conover and Munch 2002) and recruitment (Persson et al. 2007). What remains less studied, however, is the potential contribution of male body size to the stock's reproductive success and the mechanisms underlying this contribution (e.g., mate choice). In wild populations, environmental variables can generate high stochasticity in recruitment (Eckert 2003, Beldade et al. 2006) and demonstrating the effects of size truncation on population's reproductive success might be challenging. Therefore, experimental studies may be helpful showing the effects of size truncation on population's reproductive rate and further help us to understand the mechanisms behind the potential positive relationship between parental body size and reproductive success.

In addition to the population level demographic changes, fishing can also change individual life histories (Ricker 1981, Rijnsdorp et al. 2005, Vainikka et al. 2009). Life-history traits strongly influence stock productivity because they determine the timing of mortality and reproduction, thus detecting changes in life-history traits is important to the management of fish stocks. One of the principal life-history changes caused by size-selective fishing is reduced age at maturation, which has been detected by several long-term studies of exploited fish populations (Ricker 1981, Rijnsdorp 1993, Ricker 1995, Heino and Dieckmann 2008). Earlier maturation can be a result of improved growth conditions because fishing can lead to greater per capita food supply. However, decreases in age at maturation have been often accompanied by decreases in size at maturation (Ricker 1981, 1995). Because size-selective fishing increases mortality at some particular age and size, the reproductive output is typically increased before the mortality occurs. Thus, fish that mature early and at a small size have a

fitness advantage because they have an increased rate of gene input into the population before they are harvested. A life-history trait closely linked to maturation is growth, which has also been reported to respond to size-selective fishing pressure (Favro et al. 1979, Ricker 1981, Conover and Munch 2002, Edeline et al. 2007, Swain et al. 2007). Like maturation, growth is a complex process and selection imposed by fishing might affect multiple mechanisms simultaneously influencing growth (Heino and Godo 2002, Enberg 2011). Although size-selective fishing has been suggested to select for slow growth (Favro et al. 1979, Conover and Munch 2002, Edeline et al. 2007, Swain et al. 2007), predicting the consequences of fishing on growth might not always be straightforward. For example, different components of growth are subject to various selection pressures (Enberg 2011) and the benefit of decreased fishing mortality by growing more slowly (thus entering the vulnerable size class later) must be weighed against the costs of decreased fecundity (Wootton 1998) and increased natural mortality (Pitcher 1993). When fish are faced with increasingly high adult mortality, selection will favor not only earlier reproduction but also higher reproductive effort at age, at the expense of body growth (Heino and Kaitala 1999). Indeed, size-selective fishing has been shown to lead to increased reproductive investment, in terms of ovary weight (Yoneda and Wright 2004, Rijnsdorp et al. 2005, Wright 2005) but the overall reproductive success may nevertheless remain low due to the maternal and paternal -size effects on offspring quality (Marteinsdottir and Steinarsson 1998, Berkeley et al. 2004, Birkeland and Dayton 2005) that go beyond the positive relationship between female size and fecundity.

Fish stocks have collapsed in recent years, thus the potential for fisheries-induced evolution has received considerable attention (e.g., Jorgensen et al. 2007, Kuparinen and Merila 2007, Hutchings and Fraser 2008). It has been suggested by using statistical modeling that size-selective fishing can cause genetic changes in life-history traits, for example in age and size at maturation (Dieckmann and Heino 2007, Dunlop et al. 2009). Size-selective fishing has a potential to cause evolutionary changes in harvested stocks because strong fishing-induced selection is operating on a trait, which is heritable and expresses phenotypic variation within species (Jennings et al. 1998, Law 2000, 2007). However, disentangling the plastic changes from the genetic ones in wild populations can be extremely challenging as fishing represents a massive, uncontrolled experiment in a constantly changing environment (Rijnsdorp 1993, Law 2000). Direct evidence of genetic changes (i.e. changes in genotype frequencies) caused by size-selective fishing, is seldom available, thus the inference of the evolutionary effects of fishing is often based on observed changes in phenotypes, which may also reflect responses to

changes in environmental conditions (Kuparinen and Merila 2007). The lack of conclusive evidence is likely one of the reasons why considerations of fishing-induced evolutionary changes have not entered the fisheries management level. Furthermore, evolutionary changes are often thought to occur only at time scales irrelevant to fisheries management (Palumbi 2001, Stockwell et al. 2003). However, the potential for the evolutionary change caused by fishing should not be ignored because such changes are likely to occur and they can be hard to reverse (Conover et al. 2009).

Demonstrating conclusively that fishing can cause evolutionary changes in an exploited stock is one of the many issues related to the effects of size-selective fishing that are not decisively resolved. To this end, an experimental approach can be helpful in demonstrating the demographic and evolutionary risks of size-selective fishing. It can help us to answer questions, such as how does spawner body size affect reproductive behavior, reproductive output and a range of early life-history traits, which in turn can have important consequences for population dynamics (Bailey and Houde 1989, Caley et al. 1996). To increase our understanding of the effects of spawner body size on population's productivity and to increase the awareness of the largely neglected issues, such as the potential importance of male body size and female mate choice to reproductive success, I conducted experiments focused on these topics in controlled laboratory environment and used zebrafish (*Danio rerio*) as a model species. The results of these experimental studies can be considered in a fisheries framework and thus, they can help us to understand the mechanisms behind the observed changes and the extent of the effects caused by size-selective fishing practices in the wild.

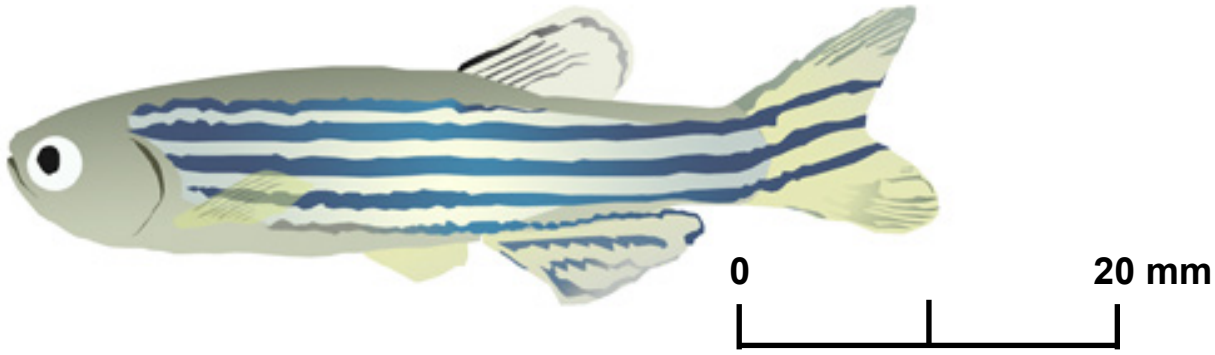
1.1 Study system

All studies in this thesis were conducted in laboratory conditions to control confounding environmental effects. Although experimental approaches have their limitations, they do have the potential to provide valuable insights into functional and mechanistic understanding of complex processes, such as reproductive success (Conover and Baumann 2009). The identical environmental conditions among treatments in each of the experiments ensured that the observed differences in reproductive success were most likely related to body size, which was the explanatory trait of interest in all of the studies, rather than to some unknown extrinsic factor. I further employed an artificial selection experiment, which can be considered a vital tool in ecological and evolutionary studies and can help us to understand the agents driving adaptation and to disentangle the correlative responses to selection (Conover and Munch 2002, Walsh et al. 2006).

Empirical model species can play an important role in advancing our fundamental knowledge of fisheries science. Although, focusing on the species with most economic importance is relevant from the management perspective, these species are often difficult to study empirically. My model species, zebrafish (*Danio rerio* Hamilton), is a small-bodied cyprinid fish native to India, Bangladesh and Nepal (Spence et al. 2008). Despite that zebrafish is used routinely in applied and fundamental research (http://zfin.org), little is known about its natural ecology and only few studies so far have been conducted on wild zebrafish (Robison and Rowland 2005, Wright et al. 2006, Hutter et al. 2010). It is not clear to what extent and to what respect laboratory strains differ from wild fish, nor how much inter-population phenotypic variation exists in nature (Spence et al. 2008). Therefore, research on phenotypic traits in wild zebrafish can be valuable itself and in particular when associated to the genetic variation. Such studies can improve our understanding of the general phenotype-genotype associations and become extremely important when related to anthropogenic disturbance, such as habitat degradation or size-selective harvesting.

In zebrafish, maturation is suggested to be related to size rather than age (Eaton and Farley 1974, Spence et al. 2008) but the age and size at maturation and the plasticity of these traits remain poorly studied in laboratory fish (Eaton and Farley 1974) and there are no studies conducted on these traits in wild zebrafish. Natural spawning is seasonal and reproduction probably cued by food availability (Spence et al. 2006) but domesticated strains spawn all

year round in laboratory conditions at intervals of 1 to 6 days (Spence and Smith 2006). Zebrafish is a batch spawner and produce relatively large eggs in small clutches over a short period at dawn (Spence et al. 2007). In laboratory zebrafish, clutch size is known to correlate positively with inter-spawning interval (Spence and Smith 2006), female age (Eaton and Farley 1974) and body size (Spence and Smith 2006) but nothing is known about the reproductive success of wild zebrafish females or males. Female mate preference and allocation of reproductive resources are determined by male body size according to some studies (Pyron 2003, Skinner and Watt 2007b), whereas other studies show that female preference is not based on male body size but on some other, unknown characteristics (Spence and Smith 2006, Hutter et al. 2010). Despite years of research in developmental genetics and embryology (Grunwald and Eisen 2002), surprisingly little attention has been paid to the life histories and mate choice behavior of zebrafish, in particular of wild zebrafish (but see Hutter et al. 2010). Ignoring the origin of the fish (i.e., whether it is a laboratory fish or a wild fish) in experimental studies can represent a fundamental shortcoming, as phenotypic, and potentially genetic, variation associated to reproductive traits, (early) life-history traits and behavioral traits expressed by laboratory strains can be extremely reduced compared to wild fish (Whiteley et al. 2011). Therefore the information received by using laboratory zebrafish may be insufficient to answer certain specific research questions.



Zebrafish, *Danio rerio*. (www.egosumdaniel.se/illustrations/Zebrafish)

1.2 Research questions

All the experiments in my thesis aimed to link body size to reproductive success by looking at this relationship from different perspectives. In addition to providing valuable new information about wild zebrafish reproductive traits and reproductive behavior, I aimed to contribute to the fundamental understanding of the potential effects of size-selective mortality

(e.g., through fishing) on fitness-related traits and its implications to populations' reproductive potential. I further present results from an artificial selection experiment and demonstrate the effects of size-selective harvesting on population's reproductive success after one generation of harvesting. In the final chapter, I show preliminary results of the phenotypic and genetic effects of size-selective harvesting after continuing the selection experiment for five generations.

The specific questions addressed, each with a corresponding research topic (Fig. 1) are:

1. **MATURATION.** Maturation is known to be a plastic process and it is often strongly related to growth. Here, I studied whether maturation exhibits plasticity in wild zebrafish and which factors determine maturation. The probabilistic maturation reaction norm (PMRN) is a statistical model, commonly used to disentangle the plastic responses from genetic ones in maturation. Despite this method has been used extensively, it has not been assessed experimentally. Therefore, I additionally asked, does the PMRN predict maturation probability accurately and account for all the phenotypic plasticity in maturation probability? (**Paper I**)

2. **MATERNAL- AND PATERNAL-SIZE EFFECTS ON REPRODUCTIVE SUCCESS.** The effects of maternal body size on offspring phenotype has been intensively studied but surprisingly little is known of the influence of paternal body size on reproductive success and offspring fitness. Therefore I asked, is the reproductive success in zebrafish mostly determined by female body size or can we expect male body size to contribute to the reproductive output and early life-history traits as well? The experimental design employed in this study implicitly recognizes the importance of free behavioral interaction between a female and a male. Similar approach has not been commonly used in earlier studies, which have typically employed artificial fertilization. (**Paper II**)

3. **FEMALE MATE CHOICE AND DIFFERENTIAL ALLOCATION.** Mate choice and reproductive behavior are closely linked to reproductive success but are often neglected in studies where the positive relationship between female phenotype and reproductive success is emphasized. I tested whether zebrafish females exercise mate choice based on male body size and whether females can be expected to allocate more reproductive resources to large males compared to small males. I further tested if the

potential allocation pattern is plastic and could be altered by manipulating the expected future mate quality. (**Paper III**)

4. **THE EFFECTS OF SIZE SELECTIVE HARVESTING.** In this chapter, I describe results of the artificial selection experiment after one generation of harvesting that demonstrate differences in reproductive success among spawning stocks consisting of large, small and random-sized fish (**Paper IV**). Finally, I introduce some behavioral aspects of size-selective fishing (**Paper V**).

5. **GENETIC EFFECTS OF SIZE-SELECTIVE HARVESTING – PRELIMINARY RESULTS.** In the final chapter, I present preliminary results answering the question: can size-selective harvesting induce phenotypic and genetic changes in an experimental fish population after only five generations of harvesting?

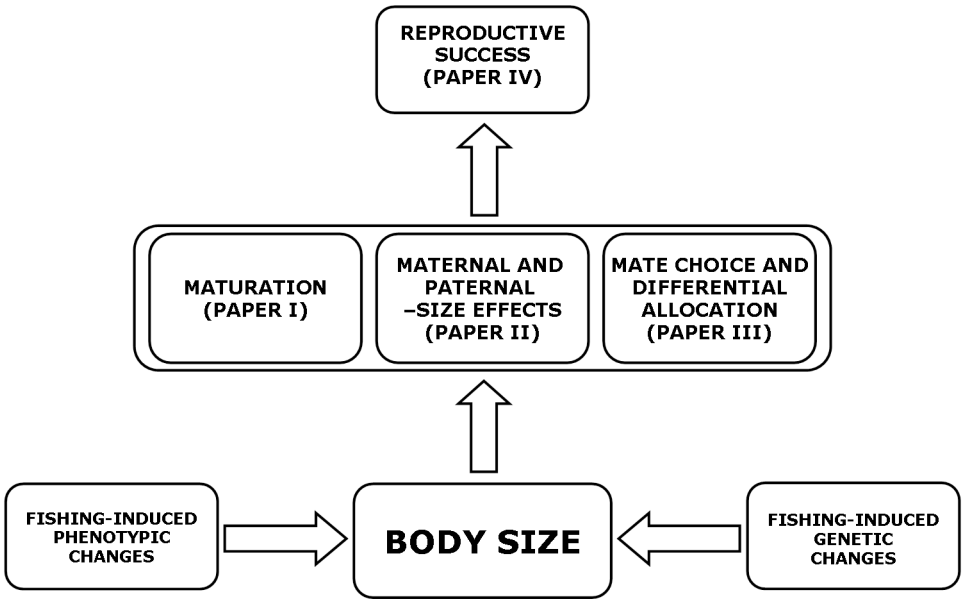


Figure 1. Size-selective fishing reduces average body size in a population and this phenotypic response can have a plastic (reversible) or genetic origin (slowly reversible or largely irreversible). Regardless of the origin, the change toward smaller body size can have serious short-term or long-term consequences translating to changes in life histories, e.g., in age and size at maturation, and in reproductive success. Changes in the overall reproductive success can be affected by changes in maternal body size, paternal body size and by male-size dependent female mate choice and differential allocation of reproductive resources.

2 METHODOLOGICAL OVERVIEW

2.1 Maturation

To study factors determining maturation (age, length, relative condition factor) in wild zebrafish, I exposed genetically similar fish to highly diverse ecological environments, simulated by varying food levels, which were expected to induce phenotypic variation in growth and maturation. I used probabilistic maturation reaction norm (PMRN) to estimate the age- and size-specific probabilities that an immature individual matures at a given time interval (Heino et al. 2002). To visualize the PMRN and its shape and position as a function of age, size and relative condition, the lengths at which the probability of maturing would be 25, 50 and 75 % were estimated using the demographic estimation method described in Barot et al (2004a).

2.2 Maternal- and paternal-size effects on reproductive success

Here, I disentangled the maternal- and paternal-size effects on wild zebrafish reproductive output and early life-history traits. Females and males were assigned into four different size-categories: small, medium, large and very large and coupled in a full factorial design. In addition to monitoring reproductive output and early life-history traits of the different size combinations, I summarized the effects of individual traits on overall reproductive success and used spawning probability, clutch size, egg fertilization probability, egg survival probability and larval hatching probability to estimate an integrative measure of reproductive fitness.

2.3 Mate choice and differential allocation

To answer the question whether zebrafish females prefer larger males, differentially allocate reproductive resources based on male body size and whether this pattern can be altered by previous experience of different-quality males, I conditioned same-sized females to social environments, which consisted of different-sized males (random-sized, large and small). After the conditioning period, individual females from different conditioning treatments were coupled with either a large male or a small male for two days and the spawning frequency, egg number and egg fertilization rate, which defined the reproductive success in this study, were monitored.

2.4 The effects of size-selective harvesting

In the selection experiment, wild zebrafish were subjected to three forms of size-selective harvesting (with two replicates for each treatment): large-, small- or random size-selective harvest. I applied 75 % mortality rate, which was chosen to mimic mortality in recreational fisheries where it has been reported to be realistic, although close to the maximum (80 %) mortality rate (Lewin et al. 2006). Populations selected for small body size were harvested of all fish with lengths exceeding the 25^{th} percentile (i.e., the largest 75 % were removed), a practice that mimics recreational and commercial fisheries. In populations selected for large body size, all fish with lengths below the 25^{th} percentile were harvested (i.e., the smallest 75 %). The control populations were harvested at a 75 % rate, but randomly with respect to size (Fig. 2). To assess the reproductive output of differently-selected spawning stocks after one generation of selective harvesting (F_1 –generation), I monitored the spawning frequency, egg numbers and fertilization rate. I further estimated several early life-history traits, such as egg size, egg mortality rate, larval age-at-hatch, larval length-at-hatch, larval area-at-hatch, larval yolk-sac volume and the effect of parental size on offspring growth. These traits were used to describe the reproductive success of the differently-selected lines.

The first five generations ($F_1 – F_5$) were all held in the rearing tanks, harvested at maturation (i.e., when 50 % of the randomly selected fish were mature) and the spawners were transferred to the spawning facility to produce offspring for the next generation. The selection continued for five generations after which it was halted for three generations. Differences in size at age and reproductive output (defined as spawning frequency and number of eggs produced) among the selection treatments were monitored for each generation. In addition, differences in age and size at maturation and in growth among the selection treatments were studied using previously published protocols (**Paper I**, **Paper IV**). For the genetic analyses I sampled F_0 –, F_1 –, F_3 – and F_6 –generation fish and used 384 non-neutral markers (single nucleotide polymorphisms), which were distributed evenly over the genome. The markers were selected from a previously analyzed wild zebrafish dataset (Whiteley et al. 2011).

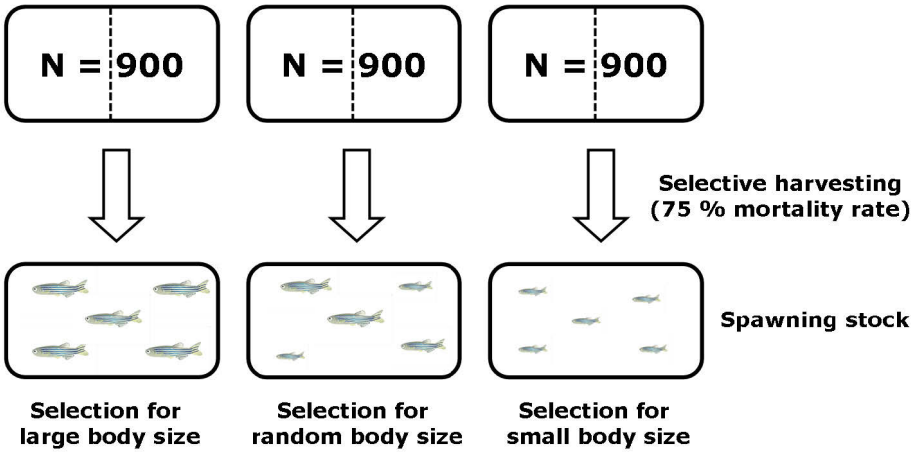


Figure 2. In the artificial selection experiment, fish were reared in six rearing tanks with two replicate each (N = 450 per replicate). 75 % mortality regime was applied when the fish started to mature. The spawning stocks were transferred to the spawning facility for the offspring production and the next generation juveniles were transferred back to the rearing tanks at age 30 days. The selection was continued for five generations.

3 MAIN FINDINGS AND DISCUSSION

3.1 Maturation

In fish, maturation exhibits tremendous amount of phenotypic plasticity (Alm 1959, Reznick 1983, Reznick 1990), which is determined by the environmental conditions individuals encounter. The most typical environmental variables which influence maturation, and growth, are food supply, temperature and light (Alm 1959, Sandstrom et al. 1995, Hansen et al. 2001). Maturation and growth are closely linked and acceleration of growth can result in earlier maturation (Policansky 1993, Atkinson 1994, Trippel 1995). However, when energy intake is low most of the necessary energy allocations may be directed to maintenance of body functions and less to somatic growth and gonad growth (Roff 1983). Thus, decreased resource availability may cause maturation to occur both later and at a smaller size, whereas when resource levels increase individuals tend to grow faster and reach maturity earlier and at a larger size (Reznick 1993). High fishing pressure is known to increase per capita resource availabilities by reducing population densities and this might result in earlier maturity at a larger body size (Stearns and Crandall 1984, Reznick 1990). Larger body size can further lead

to higher fecundity and shorter intervals between successive spawnings among the surviving individuals (Hislop et al. 1978, Reznick and Yang 1993) and collectively, to a large increase in the potential growth rate of the population. This increase in individual and population growth rate represents a compensatory response (Policansky 1993, Trippel 1995), which is typically invoked to explain earlier maturation in exploited stocks. However, one has to keep in mind, that although age and size at maturation are highly plastic and vary in response to the environment, they are also partly genetically determined (Aubin-Horth et al. 2005, Basolo 2008) and perturbations in the environment, such as size-selective fishing, may induce an evolutionary response in these traits by selecting for small body size and early maturation (Olsen et al. 2004, Swain et al. 2007).

In **Paper I**, entitled "Experimental assessment of the probabilistic maturation reaction norm: condition matters", I showed that growth and maturation are highly plastic processes in zebrafish. I demonstrated that individuals held in low-food environment had a higher probability to mature later and at smaller sizes compared to individuals held in high-food environments. In addition to length, my results suggest that condition, that represents individual's nutritional status, might be a significant factor in determining maturation probabilities. Nutritional state can strongly affect maturation (Bernardo 1993, Metcalfe 1998, Marteinsdottir and Begg 2002), for example through a certain energy threshold level, which must be reached before individual can initiate maturation (Rowe et al. 1991, Silverstein et al. 1997). This suggests that fast growing individuals attain the physiological minimum reproductive size earlier compared to individuals exhibiting lower growth rates (Rowe and Thorpe 1990, Rowe et al. 1991). In my study, the experimental fish in low-food environment potentially allocated energy mainly to maintenance of body functions, thus were less often able to reach a potential condition and / or size threshold compared to fish reared in high-food environment. This study shows that maturation in zebrafish is not only determined by length but also by condition. Altogether, my results support the general finding that when resource levels are high, individuals mature earlier and at a larger size.

One of the great challenges in fisheries science is to determine whether the changes observed in maturation are of plastic, i.e., caused by temporary changes in environmental conditions, or of genetic origin, i.e., caused by divergent selection. Heino et al. (2002) proposed to use a probabilistic maturation reaction norm (PMRN) to disentangle phenotypic plasticity and genetic effects influencing maturation. PMRN is expected to capture most of the

environmentally-induced variation in maturation, assuming all the variables contributing to maturation are incorporated in the model (Heino et al. 2002, Dieckmann and Heino 2007, Heino and Dieckmann 2008). Horizontal shifts in the position of the PMRN reflect phenotypic plasticity and a shift in the vertical position is often considered as an indication of a genetic change in the maturation schedule (Fig. 3; Heino et al. 2002, Dieckmann and Heino 2007, Heino and Dieckmann 2008). PMRNs have been estimated for numerous fish populations of marine and freshwater species, including Atlantic and Baltic cod *(Gadus morhua* Barot et al. 2004b, Vainikka et al. 2009), American plaice (*Hippoglossoides platessoides*, Barot et al. 2005), grayling (*Thymallus thymallus*, Haugen 2000), and smallmouth bass (*Micropterus dolomieu*, Dunlop et al. 2005). Many of the studies implementing PMRN suggest that changes in maturation in exploited fish stocks cannot be accounted for by growth-related phenotypic plasticity but that an evolutionary change has occurred in age and size at maturation. Most studies are based on time-series or they compare distinct periods of time but there are no studies testing the PMRN estimation method experimentally.

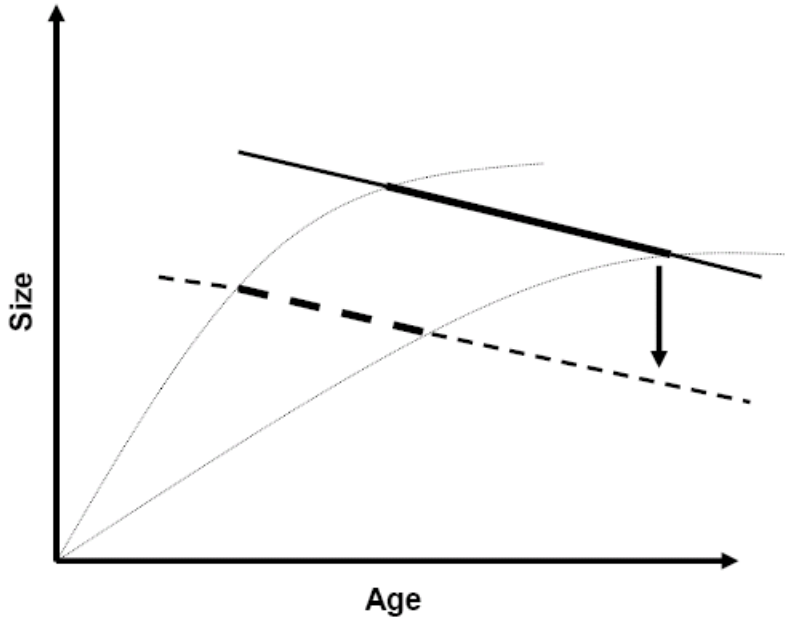


Figure 3. The principal idea of the PMRN approach. Lines indicate the hypothetical PMRN illustrating the age and size combination at which the maturation probability is 0.5. Variation in growth (growth curves in thin solid lines) determines the part of which the PMRN can be observed (thick lines). Vertical shift in the PMRN over time is not expected to be a result of growth differences but a genetic change in age and size at maturation (Heino and Dieckmann 2008). One can determine whether a

genetic change has occurred in age and size at maturation by comparing two PMRNs from different time periods.

In this study, I used the PMRN to estimate zebrafish maturation probabilities and assessed the method experimentally. As a result, not only plasticity in zebrafish maturation but also in the PMRN itself was documented (see also Morita et al. 2009). This was indicated by a diet-dependent shift in the two-dimensional, age and length based, PMRNs constructed for fish exposed to different diets (Fig. 4a). However, the shift was clearly reduced after condition factor was added in the estimation model (Fig. 4b). This indicates that condition factor is an important determinant of maturation in zebrafish. Collecting data of other phenotypic traits important for maturation than length can be challenging in the field and therefore the age and length based PMRN is often used to estimate the origin of changes in maturation for wild fish populations. However, I showed that age and length may not be sufficient for predicting maturation probabilities reliably but a more integrated view may be required. I emphasize that including other important traits, such as condition factor, in the estimation model is needed to make accurate conclusions about differences in maturation trends in the fisheries context.

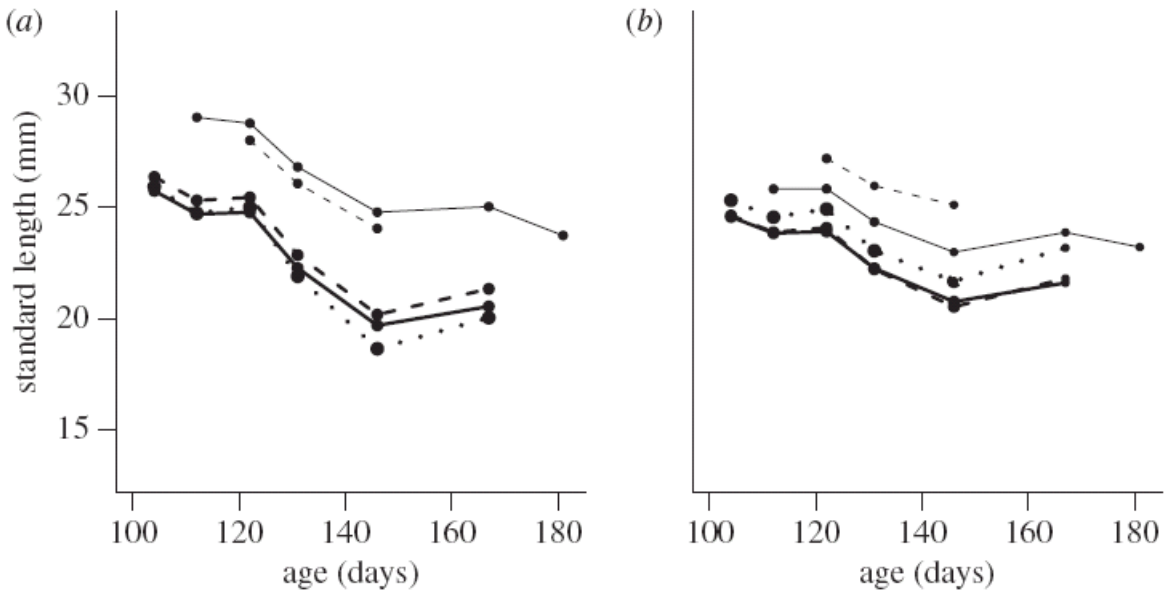


Figure 4. Probabilistic maturation reaction norms with 50 % quantiles (i.e. midpoints) for a) two-dimensional, age and length based and b) three-dimensional, age, length and condition-based PMRN models (thin dashed line: 0.5 % diet, thin solid line: 1 % diet, thick solid line: 2 % diet, thick dashed line: 4 % diet, bold dots: 8 % diet).

3.2 Maternal- and paternal-size effects on reproductive success

The effect of parental body size, in particular maternal body size, on fecundity, early life-history traits and offspring performance is widely recognized for many freshwater and marine fish species (reviewed in Wootton 1998, Green 2008, Marshall et al. 2008). Large females typically produce greater number of eggs (Wootton 1998) and often also higher quality eggs compared to small females (Chambers and Leggett 1996, Green 2008, Marshall et al. 2008). Furthermore, female body size has been demonstrated to have an effect on larval age at hatch (Kennedy et al. 2007, Regnier et al. 2010), larval size at hatch (Pepin et al. 1997, Marteinsdottir and Begg 2002), and larval condition (Berkeley et al. 2004, Sogard et al. 2008). Although large females may enhance their offspring viability through traits related to egg and larval size, such as greater feeding success, higher swimming activity and higher specific growth rate (Knutsen and Tilseth 1985, Solemdal 1997, Marteinsdottir and Steinarsson 1998), larger egg and larval sizes may also impose constraints that negatively influence survival, for example through longer developmental times (Kamler 1992) or differences in larval resource intake (Heath et al. 1999). Despite many factors suggesting that maternal-size effects positively influence offspring viability and that large females have a disproportionate contribution to the number of successful recruits, such results cannot be generalized across all fish species and ecological contexts (McLean et al. 2004, Kamler 2005), for example due to the complexity of linking the environment to the phenotype (Marshall et al. 2010).

Most of the parental-induced variation in reproductive traits is assumed to be attributable to maternal-size effects, which are propagated through the egg characteristics (Chambers and Leggett 1996, Heath et al. 1999). Maternal nutritional contribution to the fertilized egg is much greater than the paternal contribution because sperm contains virtually no extra-nuclear material and therefore it is commonly assumed that maternal effects overwhelm paternal effects (Chambers and Leggett 1996, Marteinsdottir and Steinarsson 1998, Kennedy et al. 2007). Thus, maternal- and paternal-size interactions and the contribution of sperm to developing embryo have been underrepresented in the literature and paternal effects on early life-history traits have been demonstrated only on a handful of fish species (e.g., herring (*Clupea harengus*) Hoie et al. 1999, brown trout (*Salmo trutta*), Vollestad and Lillehammer 2000, European sea bass (*Dicentrarchus labrax*), Saillant et al. 2001, haddock (*Melanogrammus aeglefinus*), Rideout et al. 2004). However, paternal body size can

contribute to the reproductive success directly, for example through male's genetic contribution to the developing offspring (e.g., good genes hypothesis; Zahavi 1975). In addition, male body size may correlate with sperm quantity or quality and this could directly affect egg fertilization rate, thus male reproductive success (Howard et al. 1998, Gage et al. 2004, Skinner and Watt 2007a). Clearly, not only maternal- but also paternal-size effects may contribute greatly to the reproductive potential of a population despite the fact that paternal-size effects are not being recognized so far as a significant source of variation in fish reproductive success. However, studying the potential effects of male body size on offspring quality and viability can be important for understanding variability in recruitment success.

In **Paper II**, "Paternal body size affects reproductive success in laboratory-held zebrafish (*Danio rerio*)", I focused on disentangling the maternal- and paternal-size effects on a number of reproductive traits and showed that large fish generally had higher reproductive success compared to small and medium-sized fish. I also demonstrated that in zebrafish maternal size correlates positively with the number of eggs but not with egg size as shown in other fish species (e.g., haddock, Hislop et al. 1978, Atlantic cod, Marteinsdottir and Steinarsson 1998, rockfish (*Sebastes* sp.), Berkeley et al. 2004). The paper further showed that maternal body size contributed significantly to larval quality, so that very large females produced larvae with larger yolk sacs compared to other-sized females. Yolk is an important energy reserve for the developing larvae and individuals with small yolk sacs might be more susceptible to starvation compared to individuals with large yolk sacs (Hunter 1981, Miller et al. 1988). The correlation between female body size and larval quality is consistent with previous studies (Marteinsdottir and Steinarsson 1998, Kennedy et al. 2007). As expected, in my study very large and large females exhibited higher reproductive fitness values compared to medium-sized and small females (Fig. 5).

The true novelty of this study lies in significant paternal-size effects identified on several larval early life-history traits. Eggs fertilized by large and very large males had higher hatching probability and shorter developmental time compared to the eggs fertilized by medium-sized and small males. Furthermore, larvae sired by large and very large males hatched at a significantly larger size compared to larvae sired by medium-sized and small males. Size is an important factor determining larval survival by increasing larval swimming abilities, reactive distances and decreasing its vulnerability to predators (Blaxter 1986), thus offspring produced by large males may have higher fitness in the wild as. The experimental

design utilized in this study, allowed free interaction between female and male instead of artificial fertilization. Therefore, I was able to observe differences in reproductive traits that were potentially related to mating behavior among different-sized fish. Interestingly, unlike among different-sized females, very large males exhibited clearly lower reproductive fitness values compared to all other male sizes (Fig. 5). Very large males did sire high-quality offspring once spawning occurred but the spawning probability and clutch size they received were substantially lower compared to other-sized males. Thus, very large body size is potentially traded off against unknown fitness costs of being too large (Fowler and Partridge 1989, Pitnick and Garcia-Gonzalez 2002) and specific behavioral processes (e.g., sexual harassment) related to very large body size (Morgan et al. 1999, Small 2004) could offer a plausible explanation to the low reproductive fitness of very large males.

Here, I identified for the first time both maternal- and paternal-size effects contributing to wild zebrafish reproductive success. Although previous studies have largely neglected the paternal contribution to offspring development, I showed that male body size may be equally important in determining reproductive success than female body size. Depletion of large females and males may have the potential to influence not only stock egg production but also the size and viability of offspring. Given the extensive effect that size-selective fishing may have on the stock's demographic structure, it would be beneficial for the fisheries management and conservation to protect and preserve both female and male size structure.

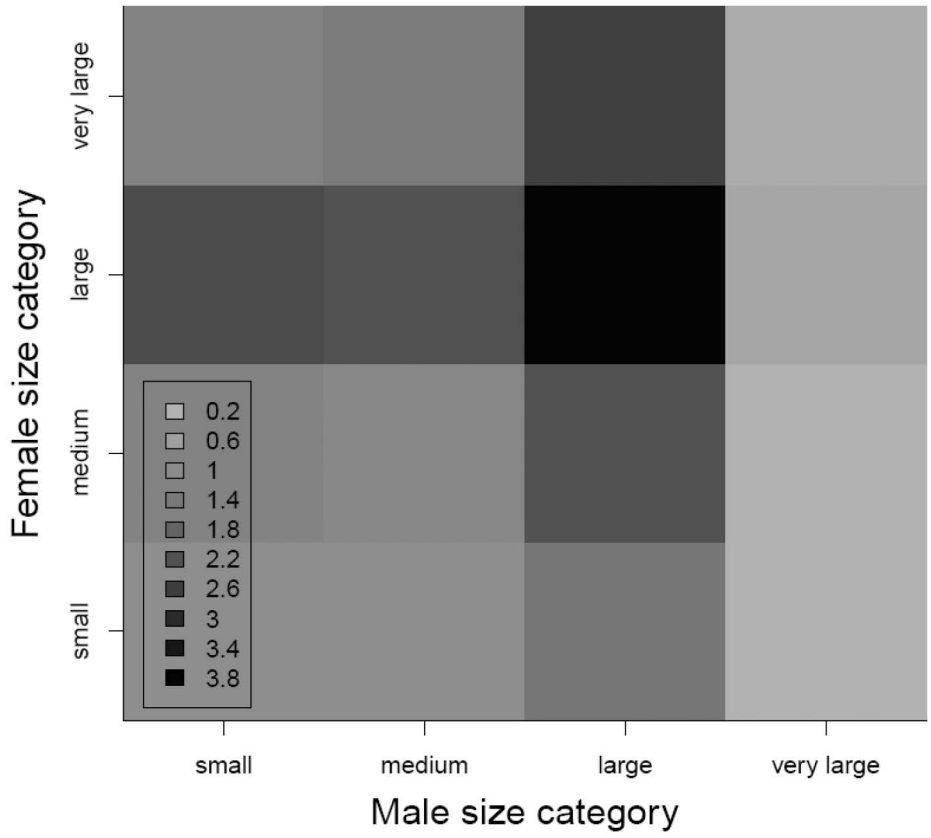


Figure 5. The integrated reproductive fitness measure (i.e., the expected number of hatched larvae) for different female and male size combinations. The values are expressed as relative to the reference size category small female : small male = 1.

3.3 Mate choice and differential allocation

Sexual selection represents selection for behavioral, morphological or physiological traits that increase individual's reproductive success (Andersson 1994) and it is partly, but not exclusively, the result of males competing for females (male-male competition) and females selecting the most attractive male to mate with (female mate choice). Female mate choice is a process leading to non-random mating with respect to one or more varying traits in males (Heisler et al. 1987). Traditionally, female choice is believed to have evolved because it provides females with direct or indirect benefits (Kirkpatrick and Ryan 1991, Kokko et al. 2003). For example, females can increase their reproductive success by choosing males that provide direct, material benefits, such as improved territory quality or paternal care (Heywood

1989, Kirkpatrick 1996). In some species, however, females do not seem to receive any direct benefits from exercising mate choice and in such cases costly female mate choice can be explained by indirect, genetic benefits to the female's offspring. Two hypotheses are traditionally used to link the female mate preference to genetic benefits: according to the sexy sons –hypothesis, the sons of choosy females inherit the male's attractive genes (Fisher 1930, Lande 1981, Eshel et al. 2000), whereas in the good-genes model, male attractiveness is thought to correlate genetically with general viability (Fisher 1930, Zahavi 1975, Moller and Alatalo 1999, Jennions et al. 2001). However, empirical evidence for indirect benefits to female mate choice is still limited (Kokko 2001, Kokko et al. 2002) and studies showing that female choice increases net offspring fitness are scarce (Kokko et al. 2003, Siepielski et al. 2011, but see Gerlach et al. 2012).

Males signal their attractiveness with various traits, such as morphological ornaments (e.g., kype and adipose fin in Atlantic salmon (*Salmo salar*), Jarvi 1990, antlers in red deer (*Cervus elaphus*), Kruuk et al. 2002), pheromones (e.g., redback spider (*Latrodectus hasselti*), Andrade and Kasumovic 2005) or behavioral characteristics (e.g., coutrship rate in guppies (*Poecilia reticulata*), Kodricbrown 1993, song duration in tree forgs (*Hyla versicolor*), Welch et al. 1998). In many fish species, male body size represents a signal of high quality. Male body size may correlate positively with the quality of material benefits he offers, for example in minnows (*Pimephales promelas*), large males build larger nests (Hudman and Gotelli 2007) and in smallmouth bass (*Micropterus dolomieui*), large males exercise more intensive parental care compared to small males (Wiegmann and Baylis 1995). Consequently, females are expected to prefer mating with larger males to increase their reproductive success, hence fitness. Females may further adjust their reproductive investment differently to different quality males (Sheldon 2000, Harris and Uller 2009). The differential allocation (DA) hypothesis predicts that females allocate their reproductive resources in favor of a relatively attractive male because of the fitness benefits associated to mating with that particular male (Burley 1988). Most of the evidence revealing DA is confined to species exercising parental care, where patterns of female investment can be explained by direct selection on female mate preferences (Kokko et al. 2003). By contrast, there are only few examples of DA by females in species that lack parental care or where males offer no material resources that can directly increase female reproductive fitness (European waterfrog (*Rana lessonae-Rana esculenta*), Reyer et al. 1999, rainbowfish (*Melanotaenia australis*), Evans et al. 2010).

Zebrafish has a resource-free mating system and males do not provide any parental care or other obvious direct resource benefits to females (Spence et al. 2008). Despite the lack of secondary sexual characteristics, females are known to exercise mate choice and prefer mating with certain males, although the traits mate choice is based on are not always clear (Turnell et al. 2003, Spence and Smith 2006). Some studies have shown that zebrafish female preference is based on male body size (Pyron 2003) whereas other studies report that male size does not determine female mate choice (Spence and Smith 2006, Hutter et al. 2010). I have shown that male body size may contribute significantly to reproductive success in zebrafish (**Paper II**) but I was not able to point out the mechanisms determining the variation in reproductive success among different-sized males. Male body size seemed to contribute to the general viability of the offspring (**Paper II**) but whether this was solely a result of male good genes or whether female mate choice and DA additionally contributed to these findings remained unclear.

In **Paper III**, ”Differential allocation in zebrafish (*Danio rerio*) is robust against the previous exposure to different-quality males”, I demonstrated that zebrafish female mate preference is mediated by male body size. This was indicated by the higher spawning probability of females coupled with large males compared to females coupled with small males (Fig. 6a). I further showed that females allocated more eggs (Fig. 6b) towards large males compared to small males, thus females expressed differential allocation (DA; Burley 1988, Sheldon 2000). There is no evidence that male body size correlates positively with sperm quality (Skinner 2004), thus egg fertilization success, in zebrafish (**Paper II, Paper IV**). Yet, I found that eggs produced to large males had higher fertilization probability compared to eggs produced to small males (Fig. 6c). This can be explained by females biasing the fertilization success by producing higher quality eggs, indicated by their higher fertilization probability (Bromage et al. 1994), to large males. Overall, the study demonstrates that zebrafish females spawned more frequently and produced a higher number and potentially higher quality eggs to large males compared to small males. My results support a small number of studies reporting similar patterns of female investment in relation to male attractiveness in fish (Hastings 1988, Kolm 2001, Skinner and Watt 2007b, Evans et al. 2010). Remarkably, this study is one of the few studies demonstrating the existence of DA in a species with a resource free mating system (Reyer et al. 1999, Skinner and Watt 2007b, Evans et al. 2010).

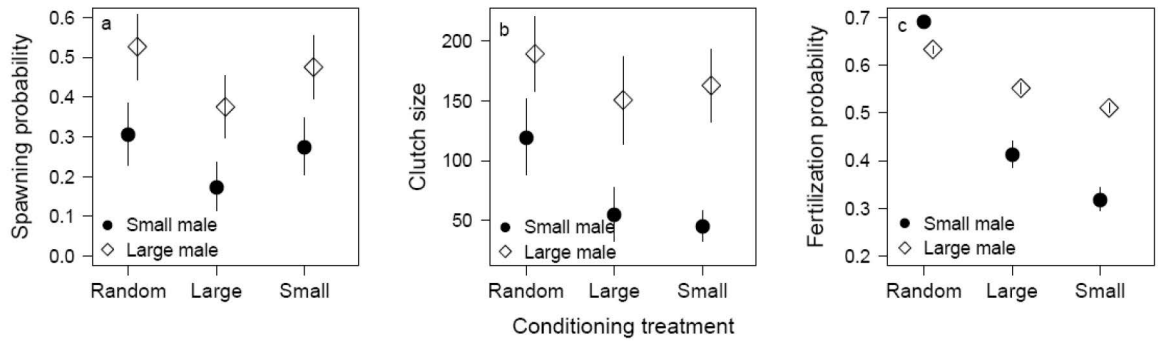


Figure 6. The a) average spawning probability, b) average clutch size per day, and c) average egg fertilization probability among females from different conditioning treatments coupled with either large or small males. Error bars indicate standard error.

Phenotypically plastic mating behavior may allow individuals to modify their mate choice or reproductive behavior to suit the prevailing environmental or social conditions (reviewed by Jennions and Petrie 1997). Indeed, it has been shown in various fish species that previous experience can influence female mate preferences (e.g., three-spined stickleback (*Gasterosteus aculeatus*), Bakker and Milinski 1991, guppy, Rosenqvist and Houde 1997, green swordtail (*Xiphophorus helleri*), Walling et al. 2008) and social learning patterns (e.g., sailfin molly (*Poecilia latipinna*), Witte and Ryan 2002, reviewed by Brown and Laland 2003). I exposed zebrafish females to different social environments consisting of different-sized males to manipulate females' expectations of the future mate quality. I showed that females did not change their mate preferences according to their social history (i.e., conditioning to different-sized males) but rather reacted to the immediate social stimuli (i.e., the size of a male they were coupled with during the spawning trial). Thus, zebrafish mate preference and allocation of reproductive resources seemed to be robust against differences in the social environment females were previously exposed to. However, females appeared to be less discriminative towards small males after conditioned to various male sizes (Fig. 6b-c). Females trained to expect variation in male size might have lower discrimination capability towards different-sized males or they might be less reluctant to discriminate small males (Rosenqvist and Houde 1997). Thus, in natural conditions, where male size varies, DA may be of less prevalence.

My thesis is hereby complemented by a study, which presents an alternative mechanism underlying the contribution of male body size to the reproductive success: indirectly through female mate choice and female differential allocation of reproductive resources. This

ecologically and evolutionarily important perspective brings more depth to the studies of female- and male-size dependent reproductive success by demonstrating that male body size may be advantageous not only from a natural but also from a sexual selection point of view. Thus, removing large males from the population may operate against both forms of selection (Hutchings and Rowe 2008) and this might substantially exacerbate the effects of fishing-induced selection on reproductive success.

3.4 The effects of size-selective harvesting

3.4.1 The effect of size-truncation on stock's reproductive success

Body size is an important attribute in many fitness-related processes and, as described in the previous chapters, it can be particularly important determinant in individual's reproductive success. Although large body size may be favored by natural selection, fishing selects against it and consequently favors small body size. Because even moderate level of fishing pressure have been suggested to truncate population's size structure (Trippel et al. 1997, Scott et al. 1999), size-selective fishing can have far-reaching ecological and evolutionary consequences for population's reproductive potential (Hutchings 2002). However, demonstrating these consequences in wild populations may be challenging and therefore an experimental approach can be helpful showing the effect of size truncation, caused by size-selective harvesting, on reproductive output and in a range of early life-history traits, which in turn can have important consequences for population dynamics (Bailey and Houde 1989, Caley et al. 1996).

In the final research paper (**Paper IV**), titled "Size-dependent reproductive success of wild zebrafish *Danio rerio* in the laboratory", I ultimately aimed to show the potential short-term effects of size-selective harvesting on reproductive success by monitoring a range of reproductive traits and, additionally, offspring growth until adulthood, after one generation of size-selective harvesting. Although, the relationship between female body size and reproductive success has been demonstrated earlier in other fish species (e.g., Atlantic cod, Marshall et al. 1998, Marteinsdottir and Begg 2002, rockfish sp. Sogard et al. 2008), this study is the first to give a full description of parental-size effects on reproductive success without artificial fertilization and describing the potential parental-size effects on offspring growth in wild zebrafish. The overall lower reproductive success of fish selected for small body size, a practice that mimics the selection in recreational and commercial fisheries, compared to fish selected for large body size, and occasionally to random-sized fish, consisted

of lower spawning frequency, lower egg number (Fig. 7a), higher egg mortality rate (Fig. 7b), slower larval developmental rate (i.e. age at hatch; Fig. 7c) and lower larval condition (Fig. 7d).

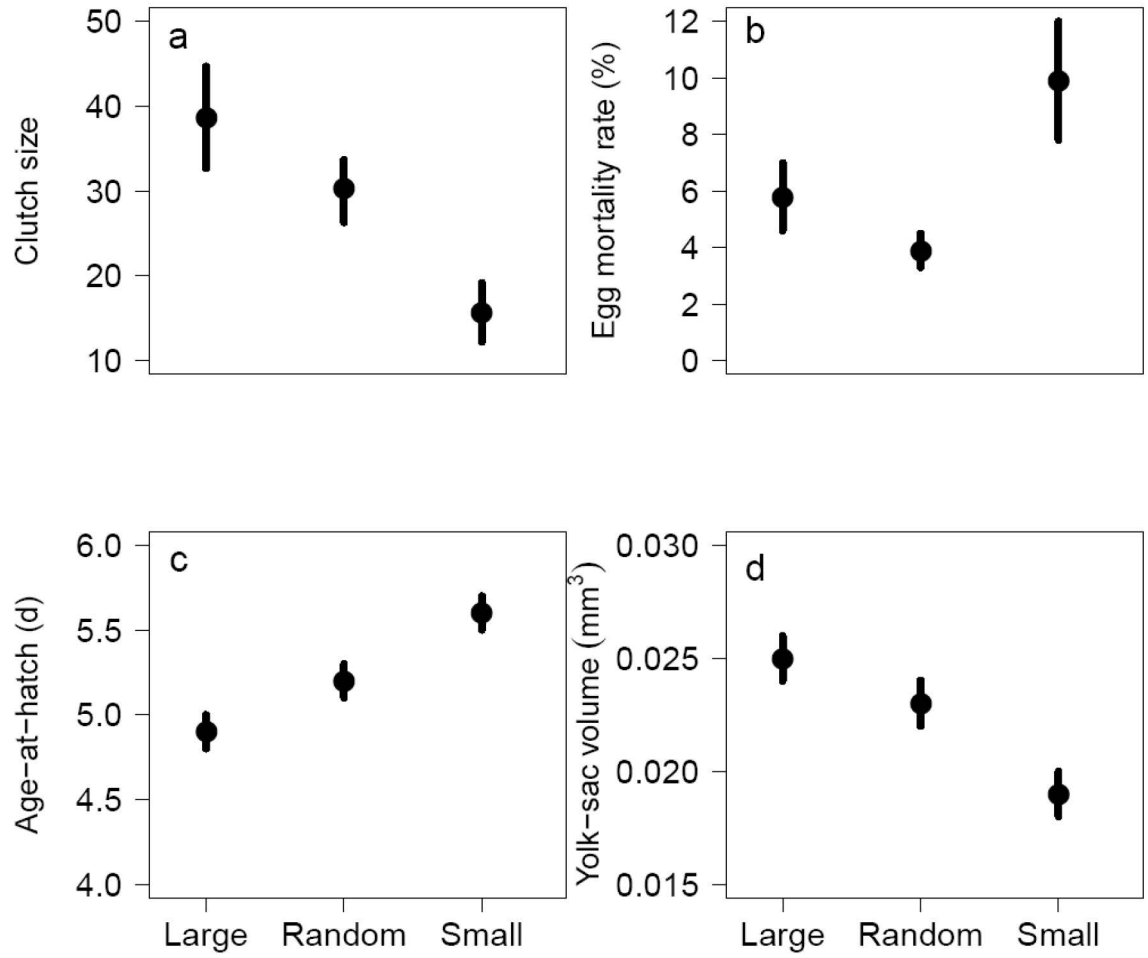


Figure 7. The average a) clutch size (i.e., number of eggs produced per female), b) egg mortality rate, c) larval age at hatch and d) larval yolk-sac volume produced by spawning stocks consisting of different-sized fish. Error bars indicate standard error.

My results indicate that depletion of large fish may seriously lower a stock's egg production and the reproductive potential is further diminished as small-sized spawners exhibit poorer egg quality compared to large spawners. Although the parental size did not have an effect on juvenile growth, the offspring of fish selected for small body size exhibited lower condition factor at adulthood compared to the offspring of fish selected for large body size and randomly.

In this experimental study, I wanted to demonstrate the effects of size truncation caused by size-selective harvesting on population's reproductive potential. Obviously, it is not possible to directly generalize experimental results to the fish stocks exploited in nature because the exact predictions of the effects of reduced parental body size on reproductive fitness also depend on environmental conditions and the demographic details of the particular species. However, an experimental study might help us to scrutinize the predictions of the relationship between parental body size and reproductive success without confounding environmental noise and to improve our understanding of the mechanisms behind this relationship. I demonstrated the importance of large fish to reproductive success and advocate studying individual reproductive characteristics to determine the level of contribution of different-sized fish to the reproductive success. Furthermore, better understanding of the effect of parental size on reproduction allows for more reliable evaluation of the consequences when the average size of reproducing fish changes as a result of, for example, size-selective fishing or stocking practices.

3.4.2 The behavioral aspect of size-selective fishing

Most of the studies of size-selective fishing are focusing on its effects on population demographic structure (e.g., Trippel et al. 1997, Scott et al. 1999) or size-related life-history traits (e.g., Ricker 1995, Swain et al. 2007). However, body size (i.e., the trait selection is operating on) may correlate genetically with physiological and behavioral traits, such as food conversion ratio and feeding behavior (Walsh et al. 2006). Consequently, selection operating on body size can be expected to operate also on the underlying physiological and behavioral traits (Fig. 8). The last paper of my thesis (**Paper V**), "A behavioral perspective on fishing-induced evolution", introduces the less studied aspect of the behavioral, and underlying physiological, responses to size-selective fishing. I point out that in passive fisheries, individual's vulnerability to capture can be largely determined by specific behavioral patterns, such as activity and boldness, rather than body size *per se*. Selection on behavioral traits can further drive changes in correlated life-history traits as active, bold and vulnerable individuals tend to grow faster compared to shy and docile fish (Walsh et al. 2006, Biro and Stamps 2008). Thus, the observed decreased average body size in an exploited stock can result from direct selection on body size or from selection on correlated behavioral traits. Studying the response in these intertwined traits to size-selective fishing could help us to understand the mechanisms underlying the observed changes, increase our understanding of the extent and

direction of fishing-induced changes and further facilitate the predictions made of the long-term effects of size-selective fishing.

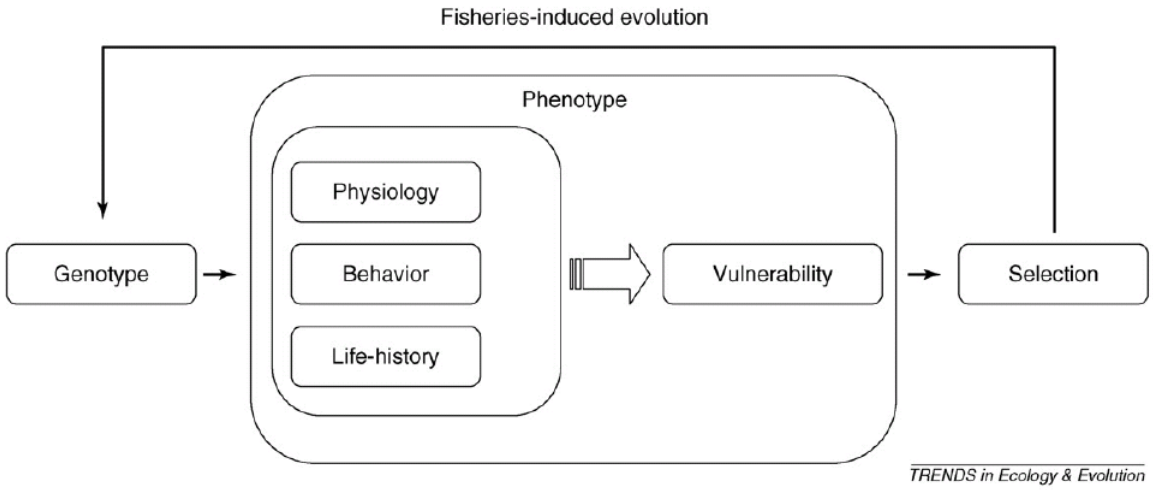


Figure 8. Mechanistic pathway of fishing-induced evolution by selection on fishing vulnerability, which can be determined by physiological, behavioral and life-history traits.

4 THE GENETIC EFFECTS OF SIZE-SELECTIVE HARVESTING – PRELIMINARY RESULTS

A question being asked in fisheries science in recent years is whether life-history responses to size-selective fishing generally are affected by phenotypic plasticity, or by changes in genotype frequencies. Theoretical modeling work suggests that only few generations of selection are required to cause an evolutionary change in an exploited stock (de Roos et al. 2006, Dunlop et al. 2007). However, such studies lack the necessary proof that the observed phenotypic changes are truly genetic and not a result of some unknown environmental factor. Conover and Munch (2002) studied the effects of size-selective harvesting experimentally by subjecting Atlantic silverside (*Menidia menidia*) populations to four generations of either large-, small- or random size-selective harvest and provided the first experimental evidence that size-selective fishing may cause genetic changes in exploited populations after only few generations of harvesting. However, their study provided merely circumstantial evidence for harvest-induced genetic changes as they lacked the molecular data which is needed to provide conclusive evidence for the potential of harvest-induced contemporary evolution.

Zebrafish genome has been sequenced and the molecular tools for the species are developed and available for use (http://zfin.org). This facilitated greatly my study of potential harvest-induced evolutionary changes as I was able to combine the traditional benefits of a model system, i.e., highly developed molecular tools, and the benefits of using a wild zebrafish that expressed genetic variability (Whiteley et al. 2011) comparable to the natural populations. After describing the effects of size truncation to the reproductive success by using the first generation fish of the selection experiment (**Paper IV**), I continued the selection for four additional generations and then halted the harvesting for all treatments for one generation. In the F_6 –generation, the fish selected for small body size were significantly smaller, in terms of standard length, compared to the fish selected for large body size or randomly. I further studied the changes in allele frequencies of selected outlier loci by using single nucleotide polymorphisms as genetic markers among the treatments and the results indicate that the populations selected for large and small body size differ genetically from each other and from the randomly-selected line, which served as a control treatment (Fig. 9).

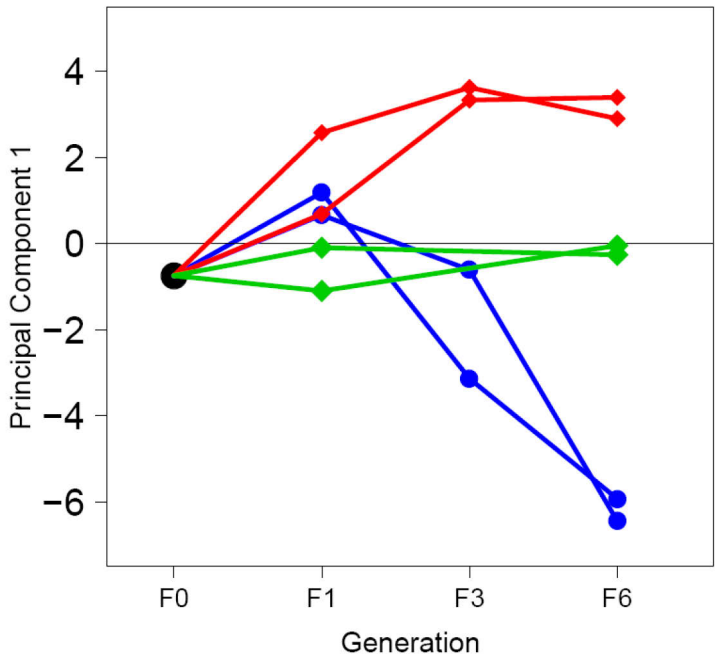


Figure 9. PC1-scores of the outlier loci (n = 27) plotted against generation (founder, F_1, F_3 and F_6-generation fish) illustrate the genetic differentiation between populations selected for large body size (indicated by blue circles) and population selected for small body size (indicated by small red diamonds). The green symbols (large diamonds) indicate the control treatment (fish selected randomly in respect to body size). Each selection treatment consists of two replicates.

After halting the selection for one more generation, I further showed that the fish selected for small and large body size differed in their growth and maturation schedules. Fish selected for small body size were significantly smaller at an early juvenile stage compared to fish selected for large or random body size but they seemed to express compensatory growth during the juvenile phase and 'catch up' the fish selected for large and random body size. However, when the fish reached the adulthood, growth of the fish selected for small body size stopped, or at least continued at a visibly decreased paste, compared to the fish selected for large body size and randomly. This could be explained by an earlier maturation among fish selected for small body size compared to fish selected for large body size. Indeed, the results from a separate maturation experiment indicate that fish selected for small body size had significantly higher probability to mature earlier and at smaller sizes compared to fish selected for large body size. After halting the selection altogether for three generations, the harvest-induced changes in body size among the selection lines were still significant. These differences in body size further translated to differences in reproductive output and fish selected for small body size had significantly lower spawning probability and they produced significantly fewer eggs during the spawning trial compared to the fish selected for large or random body size.

Here, I showed that after only five generations of harvesting populations, which evolved smaller body sizes had lower reproductive output compared to populations that evolved larger sizes. I further provided, for the first time, conclusive evidence that size-selective harvesting can cause evolutionary changes over a relatively short time period in experimental conditions. Thus, according to my results the phenotypic response to size-selective harvesting can have a genetic origin, although the applicability to the wild is not straightforward. Future studies are focusing on demonstrating the link between the observed phenotypic changes and genetic changes among the differently-selected fish by using quantitative genetics. Furthermore, more detailed genetic analyses are needed to identify potential candidate genes explaining the differences in the phenotypes among the differently-selected lines. Finally, the important questions concerning the reversibility of the genetic responses need to be answered.

5 CONCLUSIONS

In recent decades, large phenotypic changes, mostly in traits which are important for fisheries, are taking place in major fish stocks. The selective removal of large fish can reduce the fisheries yield and seriously lower the reproductive capacity of the exploited stock reducing the number of mature individuals and further decreasing the average body size of these individuals. In my thesis, I have demonstrated the importance of large females and males to reproductive success, which is further reinforced by the male size dependent female mate choice and differential allocation pattern. I have further provided conclusive evidence that size-selective harvesting may cause genetic changes in exploited populations after only five generations of selective harvesting. Regardless of the origin of the observed phenotypic changes in exploited stocks, it is clear that size-selective fishing may reduce the productivity of fish stocks by decreasing the average body size of spawners and thus their reproductive capacity. I emphasize that considerations of the role of female and male body size in reproductive success, the potential effect of mate choice and the possibility for a genetic change caused by size-selective fishing must be incorporated to the management plans. By doing so, we might be able to implement precautionary methods to reduce the deleterious effects of size-selective fishing and manage our fish stocks in a more sustainable way.

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I hereby declare that this PhD thesis has been written only by the undersigned and that no sources other than those indicated have been used. This thesis has not been submitted for a doctor's degree at any other institution. I am aware of the underlying doctorate regulations of the faculty this thesis is submitted to, i.e., Faculty of Agriculture and Horticulture of the Humboldt-University at Berlin.

Hiermit erkläre ich, die Dissertation selbständig angefertig zu haben. Ich habe mich nicht anderwärts als Doktorand beworben. Ich erkläre die Kenntnisnahme der dem Verfahren zugrunde liegenden Promotionsordnung der Landwirtschaftlich-Gärtnerischen Fakultät der Humboldt-Universität zu Berlin.

Berlin, 12.03.2012

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Silva Uusi-Heikkilä

APPENDIX

Paper I

I

Experimental assessment of the probabilistic maturation reaction norm: condition matters

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S. Uusi-Heikkilä, A. Kuparinen, C. Wolter, T. Meinelt, A.C. O'Toole and R. Arlinghaus

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Experimental assessment of the probabilistic maturation reaction norm: condition matters

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The probabilistic maturation reaction norm (PMRN) describes an individual's probability of maturing at a given age as a function of size and other relevant phenotypic traits. Population-level shifts in the PMRN are often interpreted to indicate genetic as opposed to phenotypic changes in maturation in fish. Inferences derived from trends in the PMRN have been challenged, warranting an experimental assessment of the method. This was accomplished in a laboratory experiment using zebrafish (*Danio rerio*). Fish were reared under different food levels to induce variation in growth and maturation. Plasticity in maturation was not entirely captured by the demographic age- and length-based PMRN. Adding condition to the PMRN captured a greater amount of environmental variation in maturation probability. Nevertheless, significant differences in the PMRNs among the food levels remained after accounting for the influences of age, size and condition on maturation probability indicating plasticity of the PMRN. This was particularly pronounced between fish held on low food levels as compared with fish experiencing abundant resources, with the latter experiencing higher size-specific maturation probabilities. Our analysis emphasizes the need for incorporating salient physiological traits influencing maturation, such as condition, to make accurate inferences about documented shifts observed in the position of PMRNs on maturation trends in wild fish stocks.

Keywords: growth; size-at-age; body condition; environmental variation; fisheries-induced evolution; phenotypic plasticity

1. INTRODUCTION

Intensive fishing has been proposed to be one of the main reasons for declines in size and age at maturation in exploited fish stocks [1–4]. Fishing, especially when size-selective, shifts the population's age and size distributions towards younger ages and smaller sizes through demographic truncation effects [5,6]. Moreover, when stock biomass declines owing to fishing, individual growth usually increases in response to the greater *per capita* food availability [7]. Enhanced growth rate can result in earlier maturation [8–10], reflecting an individual's response to changing environmental conditions (i.e. phenotypic plasticity). Thus, observed reductions in the average age and size at maturation in exploited fish stocks over time can be caused by demographic and/or environmental factors [5].

In addition to being phenotypically plastic traits [8,11,12], age and size at maturation are also known to be in part genetically determined [13–15]. From an evolutionary perspective, when a fish population is exposed to elevated or positive size-selective mortality, postponing reproduction for too long may be costly [2,16,17]. Thus, under conditions of high fishing mortality, individuals genetically predisposed to mature early and/or at a small size will have a higher probability of reproducing and passing on genes to the next generation than late-maturing individuals and/or individuals maturing at large size. Therefore, changes in the maturation schedule in response to increased fishing mortality can constitute either a phenotypically plastic response, an evolutionary response or a combination of both [1,2]. Because fisheries-induced evolution may have a number of undesirable consequences, such as reduced yields and speed of stock recovery [18–20], disentangling environmentally induced and genetic changes in traits affected by fishing is not only of academic interest but also of importance for fisheries management [3,4].

The probabilistic maturation reaction norm (PMRN) describes an individual's probability to mature as a function of age, size and other relevant phenotypic variables [21]. PMRN is, in principle, an individual-level property

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but in field studies a population-level PMRN is constructed [22]. This population-level approach constitutes a statistical tool which has been used to make inferences about fisheries-induced evolution of maturation schedules in wild fish stocks [22,23]. In the PMRN estimation process two assumptions are traditionally applied [21,23]: (i) most environmental effects on maturation are linked to plasticity in growth (although other traits and processes can obviously be added in the statistical model), and (ii) most environmental variation in maturation age and size is reflected by variation in size-at-age. Following these two assumptions, a PMRN is assumed to describe phenotypic plasticity in maturation probability as a function of salient traits and underlying physiological processes, such as age, size or condition [21,22]. If a population level PMRN model indeed captures all the phenotypic plasticity in maturation, the position of the PMRN itself should only change when genetic changes in age and size at maturation have occurred in response to natural and/or anthropogenic selection pressures, such as those induced by fishing (figure 1; [21,22]). Displacement of the position of the PMRN is referred to as a shift in the PMRN (figure 1).

While growth rate of individual fish certainly contributes to timing of and size at maturation [8,12], the process of maturation might be influenced by more variables than represented by age and size alone [24–26]. If the maturation process is influenced by variables not included in the statistical estimation of the PMRN, shifts in the position of the PMRN over time may not necessarily indicate genetic changes [23,27]. Indeed, shifts in the position of a PMRN in response to selection pressures in the wild might always be owing to some unaccounted for factor (e.g. condition, temperature) in the statistical model [22,23], which is why the general validity of insights about fisheries-induced evolution using the PMRN method has been challenged [3,25,28,29]. Naturally, if other processes apart from size-at-age influence maturation, the traditionally used age- and length-based (i.e. two-dimensional) PMRN method can be extended to three- or multi-dimensional PMRNs, which incorporate additional variables to control for other salient environmental covariates of maturation [30–33]. However, fisheries databases seldom contain information, which would allow resolving in detail the morphological, physiological or behavioural traits affecting maturation (e.g. weight, condition or hormonal changes that precede maturation; [34,35]). This presumably explains why many applications of the PMRN have focused on the two-dimensional, age- and length-based approach.

The ongoing debate concerning the suitability of the PMRN approach for evolutionary inference warrants for an experimental assessment of a method in a controlled laboratory setting, but so far no such study has been published. To fill this gap, we conducted an experiment under controlled environmental conditions using zebrafish (*Danio rerio*) as a model species. Genetically similar fish from one population were exposed to highly diverse ecological environments (simulated by varying food levels), which were expected to induce phenotypic variation in growth and maturation. Under these conditions and assuming that the PMRN model captures all or most phenotypic plasticity in maturation, no genetic change in age and size at maturation and thus no displacement of the PMRN midpoints (figure 1) estimated for fish exposed to different ecological environments is to be expected. Based on phenotypic information about maturity status, age, length and body condition we constructed two-dimensional (based on age and length) and three-dimensional (based on age, length, and condition) PMRNs using the demographic estimation method [36] to determine whether in zebrafish the PMRN approach fully captured the phenotypic plasticity in maturation stemming from experimentally induced variation in growth and condition.

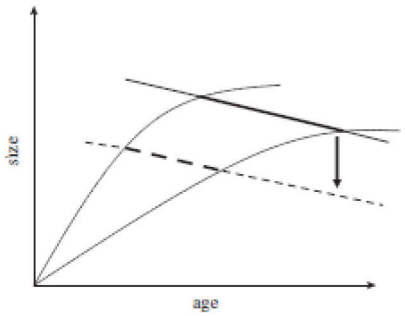

Figure 1. Schematic of the principal idea of the probabilistic maturation reaction norm (PMRN) approach. Lines indicate the midpoint of a hypothetical PMRN illustrating the combination of age and size at which the maturation probability is 0.5. Variability in growth (growth curves in thin solid lines) determines the part of the PMRN that can be observed (indicated by the thick lines). Over time, a PMRN is not expected to shift owing to differences in growth rates but a genetic change in age and size at maturation is assumed to result in its displacement [22]. By comparing two PMRNs from different time periods (the thick solid and the dashed lines), one can assess whether the data support the hypothesis of genetic changes in response to selection pressures. In this example, the PMRN shifts downwards, indicating earlier maturation at smaller size.

2. MATERIAL AND METHODS

(a) *Experimental design*

The fish used in the experiment were third-generation offspring from a wild zebrafish population captured from a river system west of Coochibar (West Bengal, India, 22.56° N, 87.67° E) reared under laboratory conditions. No obvious phenotypic traits were selected for in the experimental population and fish were assumed to have the same genetic background.

At age 85 days post fertilization (dpf) when zebrafish were still immature, a feeding experiment was initiated. This age was chosen when initiating the experiment to assure that juvenile fish were large enough to resist unfavourable environmental conditions, i.e. starvation resulting from low food amounts in some feeding treatments. Individual fish were then randomly assigned to five different feeding groups and fed with 0.5, 1, 2, 4 or 8 per cent dry food of fish biomass per aquarium per day. Food amount was adjusted throughout the experiment (see the electronic supplementary material). Each diet was applied in five replicated aquaria with 50 fish per aquarium (density of 1.1

individuals l^{-1}). Every 10–15 days, 25–50 randomly selected fish from each diet were culled and their standard length was estimated to the nearest mm and wet mass to the nearest 0.1 mg. The sample size was not fixed for each sampling period but it was adjusted to the expected maturation rate of the fish. Fish on the high food levels (2%, 4% and 8%) were detected to mature earlier and thus collected more intensively at the beginning of the experiment to obtain a representative proportion of immature and mature individuals than fish on the low food levels (0.5% and 1%). After culling, the fish were opened and the sex determined visually as described in the electronic supplementary material. Since maturity status of males could not be accurately estimated macroscopically, maturity data were collected from females only. Batch-spawning zebrafish have several oocytes simultaneously at different developmental stages when they are about to spawn [37], so females were classified as immature or mature, rather than maturing. Fish on high food levels (2%, 4% and 8%) were sampled a total of seven times from day 103 to 197 after initializing the experiment. Fish fed on 0.5 and 1 per cent diets were very small and generally had undeveloped gonads. Therefore, fish in these diet treatments were not lethally sampled as often as fish in the other diet treatments to avoid sacrificing the fish prior to them achieving the critical stage of potential maturation. As a result, female maturity data were only obtained five times from 1 per cent diet fish and three times from 0.5 per cent diet fish. Each sampling event took an average of five days. More details about fish rearing, feeding and determination of maturity status can be found in the electronic supplementary material.

(b) *Estimation of probabilistic maturation reaction norms*

The PMRN is defined in the majority of the studies by age- and size-specific probabilities that an immature individual matures during a given discrete time interval [21]. To estimate PMRNs, we used the demographic estimation method [36], which involves three steps: (i) estimation of the probability of being mature at a particular combination of variables such as age and size (i.e. estimation of maturity ogives), (ii) modelling growth rates, and (iii) estimation of the PMRNs based on the maturity ogives and the growth model. The demographic estimation method has been used in previous PMRN studies based on a simplifying assumption that the growth rate and survival is the same for immature and mature individuals. Although this assumption is not expected to apply accurately in fish [38], Barot *et al.* [36] showed that the method is fairly robust. In the current application, we estimated the maturity ogive by including the variables age and length in the two-dimensional models, and age, length and condition in the three-dimensional model. The condition factor was included because previous research has found that condition serves as a surrogate for the nutritional status of fish and may represent a valid determinant of maturation probability [30,31,39]. In our study, the condition factor was represented by the relative condition factor [40,41]. The relative condition factor was calculated for each female as

$$\frac{W}{aL^b}, \tag{2.1}$$

where W is the wet mass (g), L the standard length (mm), a the intercept and b the slope of a linear regression of $\ln(W)$ on $\ln(L)$ for females originating from the same population used in the present experiment but reared outside our experimental approach in typical feeding conditions in our laboratory ($F_{1,156} = 1352$, $p < 0.01$). The standard length–weight regression parameters of our zebrafish population were estimated as $a = 0.00003$ and $b = 2.907$.

Maturity ogives required for the calculation of the PMRNs were estimated using logistic regression. The ogive models $o(a, l)$ and $o(a, l, c)$ were estimated for the traditional, two-dimensional PMRN relating the maturation probability to age (a) and length (l) exclusively (equation (2.2)) and for the three-dimensional PMRN to age-, length- and relative condition (c) (equation (2.3)):

$$o(a, l, d) \sim a + l + d \tag{2.2}$$

and

$$o(a, l, c, d) \sim a + l + c + d, \tag{2.3}$$

where age (a) in days, standard length (l) in mm and relative condition factor (c) were continuous variables, and diet treatment (d) was a categorical variable. Additionally, the quadratic terms of age (a^2), length (l^2) and relative condition factor (c^2) were added to the models to test for non-linearity in the predicted relationships. On relatively low sample sizes the estimation of a full model may no longer be robust [36] and therefore interactions were omitted from the models. Maturity ogives were not estimated for each diet separately owing to low numbers of observations of mature fish in low food diets (i.e. 0.5% and 1% diets). This problem was surmounted by combining data from all the diet treatments as suggested in Barot *et al.* [36] and as would be typical when constructing PMRNs from phenotypic data collected in the wild. The potential variance in the probability of being mature associated with the replicate aquaria in the diet treatments were accounted for by considering replicates as a random effect in the models and estimating its variance component.

As the second step in the PMRN estimation, growth in length was modelled testing both nonlinear and linear multiple regressions with age, diet and their interaction as predictor variables. Changes in relative condition were modelled similarly by using age, length, diet and their interactions as predictor variables. As in the maturity oogive calculations, growth and relative condition models were based on data collected from females only. There were no mortalities during the experiment.

In the ogive model, age was used as a continuous variable instead of estimating ogives for each age class separately (as in Barot *et al.* [16,36]) owing to the relatively low number of females per age class. Using age as a continuous variable also helped to avoid biases in maturity ogive estimations arising from sampling design. This is because for logistical reasons sampling did not take place for each diet exactly the same day but the low food diets were always sampled a few days later than high food diets. Therefore, using age as a discrete variable would have resulted in a few extra days to mature for fish on low food diets in each sampling period. By contrast, following the commonly applied demographic estimation method [36] PMRNs were estimated for discrete age classes. The age classes i were here represented by the midpoint of the sampling period lasting on average 5 days. The PMRNs were constructed for the two-dimensional

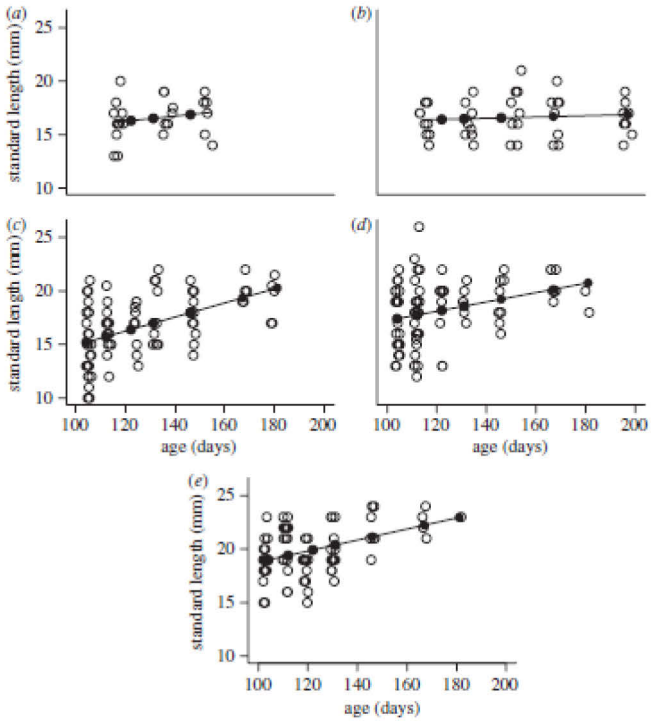

Figure 2. Growth curves for (*a*) 0.5%, (*b*) 1.0%, (*c*) 2.0%, (*d*) 4.0% and (*e*) 8.0% diet treatments in zebrafish (*Danio rerio*). Open circles indicate the observations (offset to improve the visualization) and filled circles the estimated mean values for standard length used in the probabilistic maturation reaction norm estimations. The solid lines represent growth curves predicted by the growth model (see electronic supplementary material).

length-based (equation (2.4)) and the three-dimensional (equation (2.5)) approach for each diet treatment separately using the approach by Barot *et al.* [36]:

$$m(a,l) = o(a,l,d) - o(a[i]-a[i-1], l-\Delta l)/1 - o(a[i]-a[i-1], l-\Delta l) \quad (2.4)$$

and

$$m(a,l,c) = o(a,l,c,d) - o(a[i]-a[i-1], l-\Delta l, c-\Delta c)/1 - o(a[i]-a[i-1], l-\Delta l, c-\Delta c), \quad (2.5)$$

where m refers to the probability of maturing. The probability of being mature at a given age and size was calculated from the maturity ogives, $o(a,l,d)$, $o(a,l,c,d)$, and the mean age-specific growth increments in length (Δl) and the mean age-specific changes in relative condition (Δc) were included from the final growth and condition models. To visualize the PMRN and its shape and position as a function of size, age and relative condition, the lengths at which the probability of maturing would be 25, 50 and 75 per cent were estimated using equations (2.4) and (2.5) for a range of standard lengths (10–26 mm). Following the approach by Barot *et al.* [36], a logistic regression was fitted to the estimated probabilities for the length range to derive the desired quantiles. The logistic regression model was described as

$$\text{logit}(p) = \alpha + \beta \times l, \quad (2.6)$$

where the logit link function is given by $\text{logit}(p) = \ln[p / (1-p)]$, α and β are the parameters to be estimated and l notates for standard length (mm). To test whether fish that were fed with different diets had different PMRNs, a randomization test as described by Barot *et al.* [16]. was used (see the electronic supplementary material for details). All statistical analyses were performed using R 2.10.0 with packages MASS and lme4 [42].

3. RESULTS

By the end of the experiment 37.9 per cent (122 individuals) had matured and 62.1 per cent of the females (200 individuals) remained immature. While fish on the 0.5 and 1 per cent diets exhibited minimal growth in length during the experiment, zebrafish in the high food treatments (2%, 4%, 8% diets) showed positive growth in terms of body length (figure 2). This resulted in significant differences in mean standard length of zebrafish among the various diet treatments. When pooled across the experimental period (103–197 days) fish from the 0.5 per cent (16.5 ± 1.81 mm, mean $\pm$ s.d.) and 1 per cent diet treatments (16.6 ± 1.80 mm) were on average smaller, in terms of standard length, than fish from the 2 per cent (16.7 ± 2.91 mm), 4 per cent (18.2 ± 2.88 mm) and 8 per cent diets (19.9 ± 2.21 mm). When modelling growth in length, linear and nonlinear

Table 1. Two- and three-dimensional maturity ogive models. Nagelkerke R^2-values [57] for the two-dimensional model: 0.39 and for the three-dimensional model: 0.42. AIC-values for the two-dimensional model: 282 and for the three-dimensional model: 260.

approach	variable	[a]deviance (d.f.)	[b]*p*-value	null deviance	residual deviance
two-dimensional	age^2	4.37(1,315)	0.04	427.3	268.5
	length	50.1(1,315)	<0.01		
	diet	32.0(4,318)	<0.01		
three-dimensional	age	18.8(1,319)	<0.01	427.3	252.2
	length	91.0(1,319)	<0.01		
	condition	47.9(1,319)	<0.01		

[a]Increase in residual deviance upon deletion from the full model.
[b]*p*-values from χ^2 –test.
The values for diet refer to all diet treatments.

models were virtually overlapping, therefore a linear growth model was used (figure 2). Length increased in the course of the experiment, but this was dependent on the diet treatment as indicated by a significant age × diet interaction (see the electronic supplementary material, table S1). Similar to length, pooled across the experimental period the relative condition factor was higher among fish held on 2 per cent (0.86 ± 0.15, mean $\pm$ s.d.), 4 per cent (1.03 ± 0.20) and 8 per cent (1.18 ± 0.24) diets compared with fish held on 0.5 per cent (0.76 ± 0.19) and 1 per cent (0.69 ± 0.14) diets. As in growth model, the interaction of age × diet significantly correlated with the relative condition (see the electronic supplementary material, table S1), implying that the effect of the diet on zebrafish condition differed at varying ages or stages of the experiment. Relative condition was independent of body length and not significantly related to it (see the electronic supplementary material, table S1).

The maturity ogive models used in the estimation of the two-dimensional, age- and length-based PMRN included the main effects of age, length and diet treatment. In the age- and length-based ogive model, length and the quadratic term of age were both important determinants of maturity (table 1; see the electronic supplementary material, table S2), but these did not entirely encompass the variation in maturity status among the diets. This was reflected by the significant effect of the diet treatment in the ogive model after controlling for variation in age and length (table 1). Age (ANOVA, D (deviance)$_{1,315} = 2.747$, $p = 0.097$) and quadratic length ($D_{1,314} = 0.381$, $p = 0.537$) did not significantly affect the probability of being mature. In the three-dimensional maturity ogive model the main effects of age, length, relative condition factor, and diet were included. Age, length and relative condition were found to be significant covariates of maturity probability (table 1; see the electronic supplementary material, table S2), but the effect of diet was not significant ($D_{4,318} = 8.780$, $p = 0.066$) and was therefore omitted from the three-dimensional ogive model. The quadratic effects of age ($D_{1,314} = 0.639$, $p = 0.424$), length ($D_{1,312} = 0.012$, $p = 0.912$), and relative condition ($D_{1,313} = 0.820$, $p = 0.365$) were not significant. Systematic variation among the replicates could not be detected, i.e. there was no indication of a substantial tank effect influencing the results, because the variance component of replicates was virtually zero (less than 0.0001%).

The two-dimensional, age- and length-based PMRNs for the fish exposed to medium and abundant food levels (2%, 4% and 8% diets) exhibited lower 50 per cent quantiles (i.e. PMRN midpoints) compared with the PMRNs constructed for fish held at restricted food levels (0.5% and 1% diets, figure 3*a*; 25% and 75% quantiles are presented in the electronic supplementary material, figure S3*a*,*b*). This reflected the significant effect of diet on maturity after the removal of variation in maturation probability resulting from age and length. The significant diet-dependency of the position of the PMRN indicated that the two-dimensional PMRN did not fully capture the phenotypic plasticity in maturation of zebrafish. In other words: the two-dimensional PMRN was found to be plastic to some degree. The distance among midpoints of the PMRN constructed for high and low food levels observed in the two-dimensional length-based PMRN (randomization test, $p < 0.01$) notably decreased in the three-dimensional PMRN (figure 3*b*; 25% and 75% quantiles are presented in the electronic supplementary material, figure S3*c*,*d*), which accounted not only for age and length, but also relative condition. Although the difference in the PMRN quantiles at rich and poor feeding environments was still statistically significant (randomization test, $p < 0.01$), the smaller distance among the diet-specific quantiles of the three-dimensional age, length and condition-based PMRN (figure 3) indicated that the covariates in the three-dimensional model explained a larger amount of variation in maturation probability in zebrafish. As a result the three-dimensional PMRN exhibited less plasticity in response to environmental variation induced by food availability than the two-dimensional PMRN.

4. DISCUSSION

The PMRN estimation approach has been repeatedly used to study and interpret changes in age and size at maturation in exploited fish stocks [2,16,17] despite the fact that the method has not been tested experimentally. Our assessment of a population-level PMRN approach was accomplished by exposing laboratory-held zebrafish experimentally to environments that strongly differed in food availability to induce plasticity in growth and maturation. We found that the two-dimensional, age- and length-based PMRN did not account for all the phenotypic plasticity in maturation probability in zebrafish. In particular, the diet-dependency of the position of the two-dimensional PMRN reflected environmentally

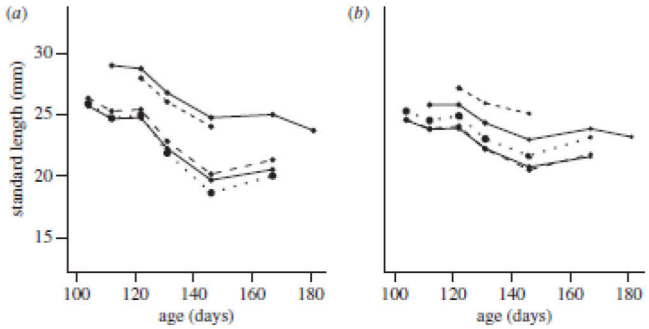

Figure 3. Probabilistic maturation reaction norms with 50% quantiles (i.e. midpoints) estimated for (*a*) two-dimensional, age- and length-based and (*b*) three-dimensional, age-, length- and relative condition-based PMRN models. Standard length on the *y*-axis represents the length at 50% maturation probability. PMRNs were estimated for the time periods data were available. In the three-dimensional PMRN the midpoints for different diets were closer to each other than in the two-dimensional PMRN in the considered scale of the *y*-axis, but, nonetheless, differences were significant (see text). The PMRNs are nonlinear owing to the age × diet interactions in the underlying growth and condition models (thin dashed line, 0.5%; thin solid line, 1% thick solid line, 2%; thick dashed line, 4%; bold dots, 8%).

induced and hence plastic, rather than genetic, variation in maturation, which was not accounted for by differential growth. However, we found that incorporating relative condition into the PMRN estimation reduced the diet-dependent differences in the vertical position of the midpoints between high and low food level treatments. Hence, the three-dimensional PMRN better explained environmental variation in maturation probability compared with the two-dimensional, age- and length-based maturation model. Adding relative condition, however, did not entirely remove the diet-dependency of the position of the PMRN, indicating that neither of the PMRN models we tested succeeded in encompassing all the phenotypic plasticity in maturation.

The assumption that variation in age and size capture most, if not all, of the environmentally induced variation in maturation probability in fish is challenged by our findings, which revealed a diet-dependency in the vertical position of the age- and length-based PMRNs constructed for zebrafish exposed to different food levels. The differences in the vertical position of the midpoints between high and low food levels were evident despite the fact that all the experimental fish were from a common gene pool, thus no genetic response in maturation schedule was possible in our within-generation experiment. Our findings agree with a study by Morita *et al.* [27], who transplanted genetically similar white-spotted charr (*Salvelinus leucomaenis*) males to five different sites in a natural river to subsequently study maturation schedules. They showed that plasticity in the position of a multi-dimensional PMRN estimated for fish exposed to different environments was caused by a habitat characteristic (river width), not by genetic changes in the maturation schedule. In our study, the midpoints of both the two- and the three-dimensional PMRNs constructed for fish exposed to rich food conditions were found to be consistently lower than the ones for the slow-growing zebrafish indicating higher size-specific maturation probabilities of fast-growing fish compared with fish growing slower owing to low food levels. These differences were evident despite statistically controlling for variation in growth and condition, but were less pronounced in the three-dimensional PMRN that included relative condition to explain maturation.

In time series from heavily exploited wild fish populations a downward shift of the PMRN has been interpreted as fisheries-induced evolutionary change towards earlier maturation at smaller size [2,16,17]. The removal of biomass through fishing usually reduces intraspecific competition for food and elevates growth rate [7], which, according to our results, may also contribute to downward shifts in the PMRN. However, in our study adding relative condition in the estimation model captured a greater fraction of the phenotypic plasticity in maturation probability than the two-dimensional PMRN based on age and length alone. This resulted in a smaller distance among the PMRN midpoints estimated for zebrafish exposed to poor and abundant food levels (figure 3). Previous field studies have similarly shown that adding condition as a third dimension in the statistical model of the maturation process improved the model fit and the accuracy of predicting maturation probabilities in fish [30–32]. Therefore, as long as researchers add all salient traits affecting maturation in fish, PMRN will capture a large degree of phenotypic plasticity in maturation. In the absence of alternative methods to disentangle phenotypic plasticity and evolutionary change in age and size at maturation, the PMRN thus remains a useful tool to study the evolutionary consequences of fishing as long as researchers carefully consider the possibility for shifts in the position of the PMRN being caused by plasticity rather than genetic change.

In the three-dimensional PMRN model, the decrease of the differences among the PMRNs estimated for the different food levels indicated that the effect of condition on maturation was more important in our experiment than has been found in previous studies, where condition only explained marginal variance in maturation probability [30–32]. This might be owing to the extreme feeding regimes in our experiment, which could have translated into larger differences in relative condition among fish than is typically seen in natural populations.

However, the occurrence of severe food limitations in nature is not uncommon and may result in stunted fish or very poor growth [43,44]. Therefore, feeding conditions comparable to those in our study could potentially also occur in nature. However, owing to the experimental set up, the importance of relative condition in explaining maturation probability was probably stronger than should be expected in the wild. For example, at the time our feeding trials were initiated, all the experimental fish were immature and of equal size. Fish cannot shrink in length even when starving, thus the different feeding treatments translated mainly into differences in an individual's relative condition. Starting the experiment earlier could have led to larger differences in body length among treatments. This could have decreased the importance of condition relative to the length in predicting maturation, but this assumption needs to be tested in the future.

One can argue that change in relative condition over time is a consequence rather than a cause of maturation (e.g. gonad weight can increase relative condition), such that there is no causal relationship between condition and maturation probability in zebrafish. Indeed, a correlative estimation method, such as the PMRN, cannot uncover the causality between the variables used for its estimation. Our study nevertheless shows that relative condition helped to explain variation in maturation probability, which was not captured by age and length alone. Condition, independent of growth rate, can represent the nutritional status of an individual [35,45] and may correlate with tissue fat level [46,47]. It has been shown that nutritional state strongly affects the maturation processes in various fish species [48–50]. It has also been suggested that an energy storage threshold must be surpassed for sexual maturation to occur in fish [46,51]. Therefore, we interpret the significant effect of relative condition on maturation probability in zebrafish in the light of its importance for affecting energy allocation towards gonad growth rather than maintenance or somatic growth. Fish continuously exposed to food limitation, as in our 0.5 and 1 per cent diet treatments, might therefore allocate energy mainly to maintenance of body functions. This might explain the higher midpoints of PMRNs estimated for zebrafish held at low food levels compared with zebrafish at high food levels, indicating lower size-specific maturation probabilities by fish experiencing restricted food conditions. To fully understand the underlying determinants of the maturation process in various fish species, a deeper understanding of the physiological processes governing maturation and how these processes respond to environmental factors and interact with growth rate is needed.

Limitations of our work are attributed to the characteristics of the model species. These limitations may restrict the implications of our findings to exploited fish stocks. Firstly, unlike many species of fisheries importance, laboratory-reared zebrafish spawn year-around on a daily basis, and thus its reproductive cycle lacks seasonality that is typical for many commercial stocks. Secondly, in many seasonally reproducing species reproductive decisions can take place early in life or during a time period before the next breeding season [35]. In zebrafish, however, maturation seems not to be determined early in ontogeny [52]. This might explain why in our study experimentally induced differences in maturation schedule were expressed even though the feeding experiment was initiated at the end of the juvenile period at 85 dpf. However, the underlying physiological mechanisms of maturation should still be similar in zebrafish compared with other fish species with a seasonal breeding cycle traditionally used in PMRN applications. A third difference among our study species and many commercially important species concerns the fact that zebrafish establish dominance hierarchies with dominant individuals controlling the feeding opportunities [53]. This may result in dominant individuals maturing larger, earlier and in better condition than subdominant fish, which might have influenced the PMRN estimation processes in the present study. However, dominance hierarchies are also common in other species, for example in cod (*Gadus morhua*) and plaice (*Pleuronectes platessa*) [54,55]. Despite the differences between our model species and other fish species, the purpose of our study was to experimentally assess the PMRN method by using a short-lived species suitable for a laboratory study. Due to the generality of the method, we do not expect substantial differences in the most important factors influencing maturation between our study system and natural fish populations.

The final limitation of the study is related to the relatively small amount of maturation data from individuals on low food levels, which might have contributed to the diet-dependency of the vertical position of the PMRN midpoints. This issue could not be avoided because fish on low food levels were consistently smaller with a lower condition factor than fish on the high food levels and had lower maturation probabilities. The low sample size of mature fish held on low food levels restricted the use of flexible ogive models with interactions between the variables so that the estimation models became biased and were no longer robust when including all two-way interactions (see [36]). Low sample size also restricted the estimation of ogive models for each diet separately. Therefore, the data collected from all diets were combined as it would be the case in phenotypic data collected from the wild allowing more robust estimation of PMRNs (e.g. [2,16]). The problem of low sample sizes in our study calls for careful evaluation of the results and upon replicating the experiment with larger numbers of observations.

PMRN estimations for wild fish stocks have often been based on length-at-age information (but see [30,31]), because sufficiently long time series of other phenotypic traits that may be important for modelling maturation are often not available [56]. However, as our study showed, age and length may not be sufficient for constructing a PMRN that is assumed to fully capture phenotypic plasticity in maturation, and a more integrated view of maturation involving indices of condition and potentially other traits may be required. This could be relevant especially among fish stocks experiencing high temporal variation in food availability, resulting in environmentally induced variation in condition among individuals. A practical implication of our study would therefore be to measure individual weight in addition to length in surveys and to estimate a condition index for maturation analyses to help providing robust inferences from PMRN analyses in wild fish stocks. There is a need to perform further assessment studies of the PMRN

method with sufficiently large amount of data to better understand the degree of plasticity that might be expected in PMRN analyses in wild fish stocks. These assessments should ideally be species-specific and conducted for commercially and recreationally exploited species, which are anticipated to be affected by fisheries-induced evolutionary changes in age and size at maturation [4].

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2. MATERIALS AND METHODS

(a) Experimental design

The first 20 days post fertilization (dpf) larvae were raised in five liter plastic boxes (length: 29 cm, width: 12 cm, height: 10 cm) in a stand alone unit with integrated mechanical and biological filters (Aquarien-Bau Schwarz, Göttingen, Germany; temperature 26.4 ± 0.7 °C; pH 8.4 ± 0.1; $N\text{-}NO^-_2 < 0.3$ mg l^{-1}; $N\text{-}NH^+_4 < 0.05$ mg l^{-1}; oxygen-level 8.1 ± 0.3 mg l^{-1}). The stand alone unit was supplied with tap water with an inflow rate of 3 ml s^{-1} and the photoperiod was set as 14 h light : 10 h dark. Larvae were fed *ad libitum* eight times a day with freshly hatched *Artemia*-nauplii and larval dry food (TetraMin; Tetra GmbH; 47.0 % protein, 10.0 % fat).

At age 20 dpf, 1250 larvae were transferred into 25 glass aquaria (volume: 45 l, length: 50 cm, width: 30 cm, height: 30 cm) in a recirculation holding facility equipped with biological filters at a stocking density of 2.2 individuals l^{-1}. Fish were kept in the aquaria for an additional 65 days prior to the start of the feeding experiment. Aquaria were supplied with tap water (water inflow rate: 14.4 ± 4.3 ml s^{-1}, temperature 27.1 ± 1.2 °C; pH 8.4 ± 0.2; $N\text{-}NO^-_2 < 0.3$ mg l^{-1}; $N\text{-}NH^+_4 < 0.05$ mg l^{-1}; oxygen-level 7.4 ± 1.2 mg l^{-1}) and the photoperiod was 14 h light : 10 h dark. Fish were fed *ad libitum* five times a day with commercial flake food (TetraMin; Tetra GmbH; 47.0 % protein, 10.0 % fat).

At age 85 dpf, a feeding experiment was initiated using the same aquaria where the larval fish were reared until reaching the juvenile stage. At this point the standard length (mean ± SD) of the zebrafish was on average 14.8 ± 2.63 mm and wet mass was 67.2 ± 37.5 mg. Individual fish were randomly assigned to five different feeding groups and fed with 0.5 %, 1 %, 2 %, 4 % or 8 % dry food of fish biomass per aquaria per day.

Each diet was applied in five replicated aquaria with 50 fish per aquarium (density of 1.1 individuals l^{-1}). Feeding was conducted five times a day and the food amount was adjusted every 10-15 days by weighing the fish and calculating new food amounts based on the biomass of fish per aquarium. At sampling, the maturity status of each female fish was determined by staining the oocytes in a solution of 96 % ethanol, 40 % formalin, 100 % acetic acid (in a ratio of 6:3:1) and observing the nucleus' position in relation to the periphery of the oocyte. A female was classified as mature when at least one egg with a nucleus migrating towards the periphery was found among all the eggs in the gonads. This is a well-established method for determining the maturity status of female fish (e.g., Selman *et al*. 1993). At age 197 dpf all the fish were culled and the experiment was concluded.

(b) Randomization test

A randomization test was conducted to test whether fish that were fed with different diets had different PMRN quantiles (Barot *et al*. 2004a). Diets were permutated randomly among females for each age class. This led to the creation of a new data set in which any difference among the maturation probabilities of fish from different diet treatments would only arise by chance. This step was repeated 1000 times. The likelihood-ratio test was used to test the significance of the diet effect on the possible shift in the intercept of the PMRN. The G^2-statistic of the likelihood-ratio test is described by

$$G^2 = -2(\ln L_R - \ln L), \qquad (4.1)$$

where $\ln L$ is the log-likelihood for the hypothesized model with all the variables (age, length and diet; age, length, condition and diet) and $\ln L_R$ is the log-likelihood for the hypothesized model when diet has been removed. The same calculations were applied to the original data without randomization and the randomized data sets. Diet was then considered to be a

significant determinant of the PMRN if less than 5 % of randomization for each age class led to higher values of the test statistic (G^2-values) than the one computed for original data.

Table S1. Final growth and condition models with their covariates and F-statistics.

Model	**Variable**	**F-value (df)**	***P*-value**
Growth	Age × Diet	3.63 (4,317)	< 0.01
Condition[a]	Age × Diet	2.70 (4,311)	0.03

[a] Non-significant, thus omitted covariates: Length × Diet ($F_{4,311} = 1.85$, $P = 0.12$), Age × Length ($F_{5,312} = 1.56$, $P = 0.17$), Length ($F_{5,313} = 0.30$, $P = 0.59$).

Nagelkerke R^2-values [57] for the growth model: 0.36 and for the condition model: 0.48.

Table S2. Maturity ogive models with their estimated coefficients and standard errors (SE) in zebrafish (*Danio rerio*).

Approach	**Variable**	**Estimated values (SE)**	***P*-value**[a]
Two-dimensional	Intercept (0.5 % -diet)	-13.5 (1.81)	< 0.01
	Age^2	0.0001 (0.00003)	< 0.01
	Length	0.48 (0.08)	< 0.01
	Diet 1 %	-0.17 (1.23)	0.888
	Diet 2 %	2.36 (1.08)	0.030
	Diet 4 %	2.52 (1.09)	0.020
	Diet 8 %	3.25 (1.09)	< 0.01
Three-dimensional	Intercept	-20.5 (2.39)	< 0.01
	Age	0.03 (0.01)	< 0.01
	Length	0.61 (0.08)	< 0.01
	Condition	15.3 (2.54)	< 0.01

[a] *P*-values derived from Wald z-tests.

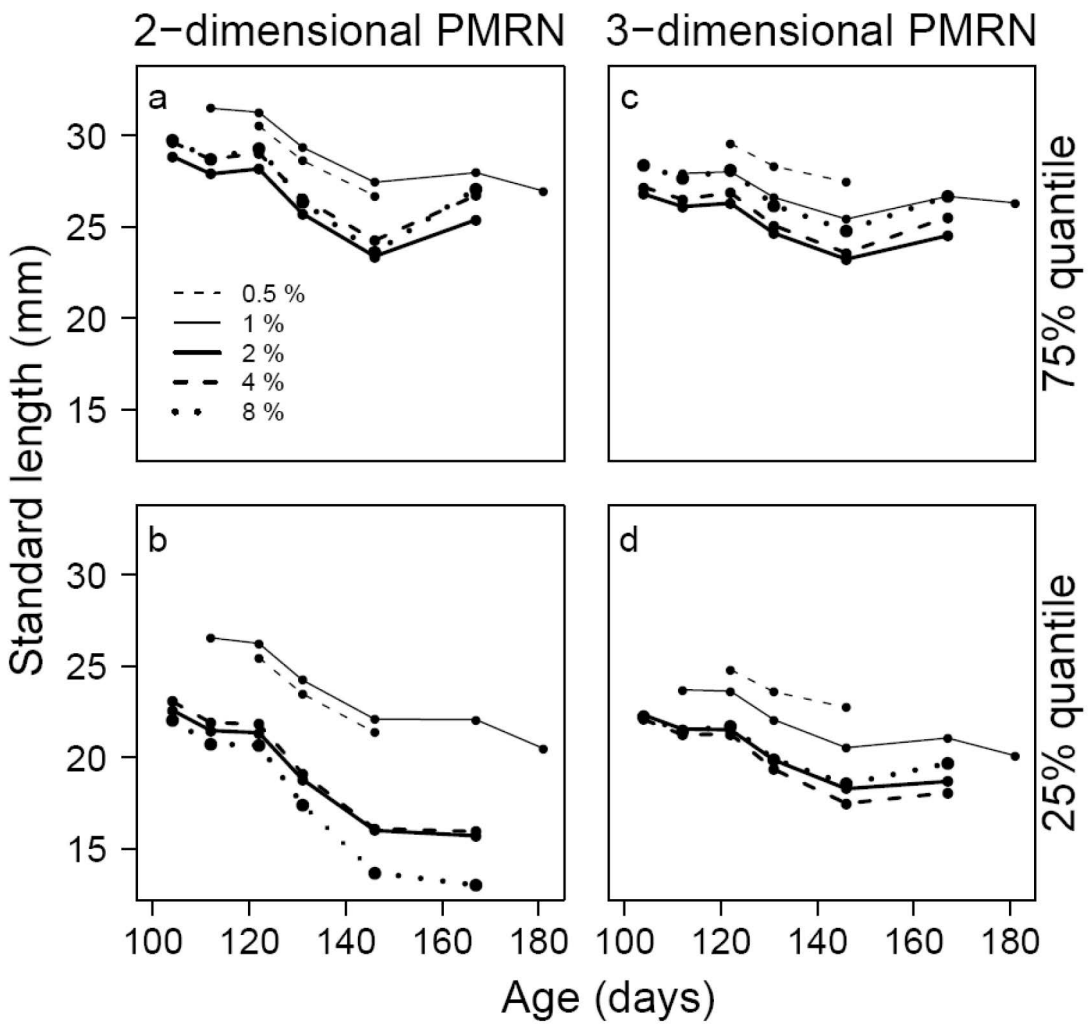

Figure S1. Probabilistic maturation reaction norms with 75 % (*a,c*) and 25 % quantiles (*b,d*) estimated for two-dimensional, age- and length-based (*a,b*) and three-dimensional, age-, length- and relative condition-based (*c,d*) PMRN models. Standard length on the y-axis represents the length at 75 % and 25 % maturation probability. PMRNs were estimated for the time periods data was available. The PMRNs are non-linear due to the age × diet –interactions in the underlying growth and condition models.

Paper II

II

Paternal body size affects reproductive success in laboratory-held zebrafish (*Danio rerio*)

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Paternal body size affects reproductive success in laboratory-held zebrafish (*Danio rerio*)

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Abstract Across many fish species, large females tend to exhibit higher individual reproductive success due to elevated fecundity and the provisioning of better conditioned eggs and offspring compared to small females. By contrast, effects of paternal body size on reproductive success are less well understood. We disentangled the maternal- and paternal-size dependent effects on reproductive output and early life history in zebrafish (*Danio rerio*). In the laboratory, females and males from four size categories (small, medium-sized, large and very large) were allowed to spawn freely in a full factorial design with 10 replicates per size combination. As expected, larger females produced more eggs and better conditioned offspring compared to smaller females. Male body size further contributed to zebrafish reproductive success: offspring sired by large males exhibited higher hatching probability and these offspring also hatched earlier and larger than offspring fertilized by small males. However, the largest males experienced lower mating success and received fewer eggs than males of the smaller size classes. While male body size substantially affected reproductive success in zebrafish, it remained unclear whether and to what degree direct paternal effects (e.g., related to sperm quality) or indirect paternal effects stemming from differential allocation patterns by females were the mechanism behind our findings. Answering this question constitutes an important future research topic.

Keywords Maternal effect · Paternal effect · Reproductive fitness · Reproductive success

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Introduction

Females are known to invest more resources into an embryo relative to males whose contribution if often

confined to sperm only, thus it is commonly believed that a progeny's phenotype is more strongly influenced by the female's phenotype than by the phenotype of the male (Chambers and Leggett 1996; Heath et al. 1999). Any effect of maternal phenotype on the offspring's phenotype is referred to as maternal effect (Bernardo 1996; Mousseau and Fox 1998). The positive relationship between female body size and offspring performance is supported by findings according to which large females of many marine and freshwater fish species spawn greater numbers and often larger eggs and larvae compared to their smaller-sized conspecifics (reviewed in Wootton 1998; Green 2008; Marshall et al. 2008). However, such results should not be prematurely generalized across fish species and ecological contexts (McLean et al. 2004; Kamler 2005; Marshall et al. 2010). In fact, larvae hatching from eggs produced by large females may also be smaller than larvae hatching from eggs produced by small females due to the size-dependent variance in hatching time and differences in larval resource intake (Heath et al. 1999). According to the fundamental life-history trade-offs (e.g., trade-off between egg number and size; Stearns 1989; Roff 2002) it is unlikely that large females can maximize all reproductive traits simultaneously. Thus, the often-cited positive influence of maternal size on offspring performance and reproductive success may not always apply in nature (Marshall et al. 2010).

Relative to maternal-size effects, the effects of paternal body size on reproductive success may be less pronounced and it has also been studied less intensively (Chambers and Leggett 1996). Males' contribution to offspring development has not been assumed to be as distinct as that of females' because males do not provide any extra-nuclear material or nutrition to the developing offspring (Marteinsdottir and Steinarsson 1998; Kennedy et al. 2007). However, paternal-size effects can operate directly, either through genetic contribution to the developing offspring (e.g. 'good genes' –hypothesis; Andersson 1994) or via physiological and energetic pathways. For example, larger males may have larger testis and longer spermatozoa with higher motility, which may elevate fertilization rates (Gage et al. 2004), compared to small males (e.g., Howard et al. 1998; Skinner and Watt 2007a). Furthermore, the effect of male body size on reproductive success may be indirectly expressed by increased female reproductive investment when mating with a high quality (e.g., large) male (Howard et al. 1998; Kolm 2001). The advantage female gains by investing more reproductive resources towards high quality male (known as differential allocation; Burley 1988) is thought to be associated with the prospects these males offer to the female's fitness, such as better oviposition sites or more intensive parental care in nest-guarding species (e.g., Sabat 1994).

Much of the previous research focused on detecting parental-size dependent effects on reproductive success in fish has been conducted by using artificial fertilization experiments in the laboratory (e.g., Chambers et al. 1989; Marteinsdottir and Steinarsson 1998). This inhibits sexual selection, mate choice and differential resource allocation patterns to be expressed, potentially biasing study findings regarding to maternal and paternal-size effects on reproductive traits (Thériault et al. 2011). To address this issue, a model species, which allows individuals to spawn freely and express mate choice may be useful. We used zebrafish (*Danio rerio*, Hamilton) to investigate maternal and paternal-size dependent effects on reproductive success using natural spawning events without artificial insemination in a full factorial design. We defined reproductive success as a combination of important reproductive traits, such as spawning probability, clutch size, egg and larval size, embryo survival and hatching probability.

Zebrafish is a small-bodied, batch-spawning cyprinid species, which spawn all year round under laboratory conditions (Spence and Smith 2006). Domesticated strains spawn at intervals of 1 to 6 days, and clutch size is known to correlate positively with inter-spawning interval (Spence and Smith 2006), female age (Eaton and Farley 1974) and body size (Spence and Smith 2006; Uusi-Heikkilä et al. 2010). In addition, female reproductive success may correlate with male body size as females have been shown to prefer (Pyron 2003) and differentially allocate eggs towards larger males if exposed to large and small males in a short sequence (Skinner and Watt 2007b). However, other zebrafish studies have not reported female mating preference towards large males (Spence and Smith 2006; Hutter et al. 2010), and ultimate female mate choice may be related to other visual (Hutter et al. 2010) or olfactory cues (Gerlach and Lysiak 2006). In addition to mate preferences, sex-ratio and population density have been shown to

affect zebrafish mating behavior and reproductive success (Spence and Smith 2005; Spence et al. 2006). High density and biased sex-ratio may lead to increased levels of aggressive interactions among males, which can have a negative effect on female egg production (Pritchard 2001; Spence and Smith 2005; Paull et al. 2010). Although parental-size dependent effects on zebrafish mating success, reproductive output and early life-history traits have been demonstrated earlier in trials comparing spawners composed of similarly-sized individuals (Uusi-Heikkilä et al. 2010), the contribution of either maternal or paternal-size effects and their interaction on reproductive success remains obscure. The objectives of the present study were to investigate whether the higher reproductive success of large zebrafish spawners relative to small spawners (Uusi-Heikkilä et al. 2010) is determined mostly by female body size or whether the variation in reproductive success is also related to male body size or the interaction between female and male body size. We hypothesized that both female and male size contribute to the reproductive success in zebrafish but the effect of female body size on early life-history traits was expected to be greater than the effect of male body size.

Materials and methods

Fish holding conditions

Our experimental fish were third generation offspring from a wild zebrafish population captured from a river system 70 km west of Coochbihar (West-Bengal, India, 22.56°N, 87.67°E). Fish were raised in six glass fiber - polyester tanks (diameter: 79 cm, height: 135 cm, volume: 320 l) in a light (14 h light: 10 h dark) and temperature controlled (26.8±0.79°C, mean ± S.D.) recirculation facility with an inflow rate of 0.25 $l s^{-1}$. The recirculation system was run with insipid tap water, and the water quality was controlled weekly for pH (8.4±0.1), nitrite ($N\text{-}NO_2^-$; < 0.3 mg l^{-1}), ammonium ($N\text{-}NH_4^+$; < 0.05 mg l^{-1}), and daily for oxygen levels (7.9±0.6 mg l^{-1}). The stocking density per holding tank was 0.9±0.2 individuals l^{-1}. We fed fish with freshly hatched *Artemia*-nauplii (Inve Aquaculture NV, www.inve.com) and commercial flake food (TetraMin, Tetra GmbH, www.tetra.net; 47% protein, 10% fat) ad libitum. Fish were fed five times per day with small amounts of food as it has been shown to result in an efficient feed utilization and to maximize growth and reproductive output (Priestley et al. 2006).

At age 250 days post fertilization (dpf), females and males were caught using a dip net. Zebrafish start maturing at age 90 dpf (Schilling 2002) and at standard length of about 19 mm (Uusi-Heikkilä et al. 2011) so by the time our experiment was initiated all fish were mature. We measured females and males for standard length (SL) to the nearest mm and then assigned them into four different size categories: small (24–25 mm), medium (26–27 mm), large (28–29 mm) and very large (30–31 mm). The size ranges were based on a preliminary experiment, where females below 24 mm were found having an extremely low reproductive success, and fish above 31 mm were rare in our experimental populations. We coupled females and males from different size categories with each other employing a full factorial design and consequently produced 16 different size combinations of females and males, each replicated 10 times (altogether 160 couples). This full factorial design allowed us to disentangle the size-dependent female and male contributions to reproductive success.

By the time the experiment was initiated, spawners (i.e., mature females and males) were transferred into a standalone spawning facility (Aquarien-Bau Schwarz, 37081 Göttingen, Germany, www.aquaschwarz.com; temperature 26.7±0.64°C; pH 8.4±0.1; $N\text{-}NO_2^-$<0.3 mg l^{-1}; $N\text{-}NH_4^+$< 0.05 mg l^{-1}; oxygen-level 7.9±0.4 mg l^{-1}) in spawning boxes designed to prevent egg cannibalism by separating adults from eggs as previously applied by Uusi-Heikkilä et al. (2010). A grid of a mesh size of 2×2 mm was inserted inside of each spawning box (volume 3 l, length: 21 cm, width: 11 cm, height: 13 cm). Each box was additionally equipped with green plastic filter material serving as a spawning substrate. Spawning boxes were stocked with one female and one male. Reproductive success of the fish from the four different size categories was assessed for four consecutive days. Zebrafish are known to spawn every 1–6 days (Spence and Smith 2006), thus it was likely that each couple willing to spawn reproduced at least once during the 4 days spawning period.

Reproductive traits

Reproductive output

Zebrafish usually spawn within the first few hours after sunrise (Hisaoka and Firlit 1962), thus the assessment of reproductive output took place between 0800 and 1000 h. During the 4 days spawning period, we cleaned the spawning boxes each morning, assessed the occurrence of a spawning event and counted the number of eggs spawned per female per one spawning event. For assessing the egg fertilization probability, we distinguished fertilized eggs from unfertilized eggs. Zebrafish eggs are translucent, and fertilized eggs can be easily identified by the presence of a multi-cellular blastodisc, which is not present in unfertilized eggs (Kimmel et al. 1995). Only clutches larger than 30 eggs were used in the egg fertilization probability estimation to avoid inflated egg fertilization probability estimates due to random egg mortalities in very small clutch sizes.

Egg traits

Egg trait measurements included the assessment of egg size and egg mortality rate. We measured egg size as egg yolk diameter. Yolk size can be a better indicator of the egg quality than egg size (Kamler 2005) because perivitelline space is not contributing substantially to the egg quality (Alderdice 1988). The eggs were photographed and the yolk diameter was measured from the photographs under a profile projector (Quick Scope; AT112-220 F; Mitutoyo; www.mitutoyo.co.jp) with an accuracy of 0.1 μm. Eggs for size measurements were selected from the first clutches spawned and these eggs were only used for size measurements, not for the subsequent analyses.

Post-fertilization egg survival was estimated from the first clutch females spawned. Egg quality can decrease in the course of spawning duration (Paull et al. 2008; Uusi-Heikkilä et al. 2010) and therefore eggs only from the first, and presumably highest quality, clutch were collected. From each female's first clutch, we transferred 15–48 fertilized eggs (depending on the total amount of fertilized eggs produced per couple) into a 24-well Multiwell Plates (BD Falcon; nontreated polystyrene; Jacob et al. 2007) so that one egg per well was incubated in 2 ml of tap water. Consequently, eggs were not influenced by other eggs or their contaminants and could be treated as independent data points in the statistical analyses. After adding eggs to the plates we transferred the plates into a rearing incubator (Tintometer GmbH, 44287 Dortmund, Germany, www.tintometer.de) at 27°C. Water in the wells was not changed during the incubation (Jacob et al. 2007). The cell well plates were controlled during the next 48 h for the egg mortalities, which were estimated by counting the number of dead eggs from each plate.

Larval traits

Larval traits for the different sized parents were assessed as larval age-at-hatch, larval length-at-hatch, and larval yolk-sac volume. Larval traits, similarly as egg traits, were assessed from the first clutches spawned. Larvae were hatching in the cell well plates and the number of larvae hatched was recorded each day during 7 days. The standard length of each newly hatched larva was measured under the dissection scope. Larvae of age 4 dpf were used to compare the larval length-at-hatch between the different sized couples. Measures based on larval length, however, may not be a reliable indicator of the quality of the larva (Kamler 2008). Therefore, we photographed individual larva to measure larval yolk-sac volume as an indicator of larval energy resources (Kamler 2008). We used the digitizing software Image Tool for Windows (version 3.0) to measure the height and width of the yolk-sac from the photographs. The yolk-sac volume was estimated using the following formula (Chambers et al. 1989):

$$V = \pi/6LH^2,$$

where L represents the length (horizontal measurement; mm) and H the height (vertical measurement; mm) of the yolk-sac.

Statistical analyses

Due to non-normally distributed and heteroscedastic data, we used generalized linear models (GLM; Crawley 2007) to disentangle maternal and paternal-size effects on reproductive output and early life-history traits. In all the analyses, female and male size categories and their interactions were treated as fixed effects. Due to the fact that zebrafish establish

potentially body-size based dominance hierarchies (Pritchard 2001; Paull et al. 2010) and the effect of these hierarchies on zebrafish mating success and reproductive output is largely unknown, we additionally tested the effect of the relative size difference between female and male on all the reproductive traits. The individual couple was set as a random variable to account for the fact that other parental traits than body size could contribute to the differences in reproductive success among couples (Spence and Smith 2006; Hutter et al. 2010). Spawning day was also treated as a random variable when estimating the effects of parental body size on variables measured over the whole experimental period (i.e., spawning probability, clutch size and fertilization probability). The amounts of variance associated to the random variables were estimated through variance components. Couples which did not produce any eggs during the four days spawning period were excluded from the clutch-size analysis. Count data, such as clutch size and age-at-hatch, were modeled using Poisson regression. All probability data (i.e., spawning probability, egg fertilization probability, egg survival probability and hatching probability), were modeled using binomial regressions. In the analyses of larval age-at-hatch, larval length-at-hatch and larval yolk-sac volume, egg size could not be treated as a covariate because the eggs measured were not the same eggs from which the larvae hatched. Using an average value of egg size per couple as a covariate in these analyses was not feasible due to the low number of observations per couple for which both egg and larval traits were measured. Instead, we did a simple correlation analysis (Pearson's correlation) between the average egg size and the average larval age-at-hatch, length-at-hatch and yolk-sac volume. If data was over-dispersed, the quasi-Poisson or quasi-binomial distributions were used to account for the overdispersion. To estimate differences among size categories we first fitted the full model and then used the stepwise model reduction that in our case referred to aggregating size categories, which had most similar response variable values with each other.

To summarize the effects of individual traits on overall reproductive success we used spawning probability, clutch size, egg fertilization probability, egg survival probability and larval hatching probability as components to estimate an integrative measure of reproductive fitness (e.g., Mousseau and Roff 1987; Danzmann et al. 1989). The components (i.e., the coefficients for each size combination predicted by the model) were multiplied to obtain the expected number of hatched larvae, i.e., our fitness measure was obtained by multiplying the model-based probabilities that an egg survives and hatches (as a product of spawning probability, egg fertilization probability and hatching probability) further multiplied with the predicted number of eggs for each size category of either males or females. This measure describes the effective offspring production as predicted by the statistical models and is not to be confused with lifetime fitness. The final fitness values are given as relative values where the values of different size combinations are standardized by the average value for the small female : small male size combination. In other words, this chosen reference value is used as a value of 1 and all other values are relative to this reference. Our final integrative measure of reproductive success described the expected number of hatching larvae, which was considered a proxy of fitness, as a function of female and male size, expressed relative to the small female : small male reproductive fitness.

All data were considered statistically significant at $P<0.05$. All statistical analyses were performed with R 2.11.1 with packages MASS and lme4 (R Development Core Team 2009). Data are presented as mean values with standard errors (SE).

Results

Reproductive output

The spawning probability was not affected by female body size whereas male body size had a significant effect on the female's probability to spawn (Table 1). Females from all size categories had a significantly lower probability to spawn with very large males (0.17±0.03) compared to mating with large (0.50±0.04), medium-sized (0.38±0.04) or small males (0.42±0.04; Table 1). The interaction between female and male size and the relative size of males and females did not affect the probability to spawn (Table 1). Spawning day captured a relatively small amount of variance (5.5%) not explained by the parental body size, while the individual couple was responsible for relatively large amount of variance (72.9%) in terms of spawning probability.

Table 1 The effects of female, male, female × male body size and relative size difference between female and male on zebrafish reproductive traits. Estimated values are given for the significant covariates, which are indicated in bold

Trait	Variable	Parameter values (SE)	χ^2-value[a] (df)	*P*-value[b]
Spawning probability	Female		0.648 (10,7)	0.886
	Male			
	Small (Intercept)	−0.608 (0.110)	20.13 (5,4)	0.000
	Medium	−0.198 (0.131)		
	Large	0.631 (0.129)		
	Very large	−2.013 (0.140)		
	Female × Male		5.008 (19,10)	0.834
	Size difference		2.276 (12,10)	0.321
Clutch size	**Female**		20.07 (9,4)	0.001
	Small (Intercept)	3.349 (0.299)		
	Medium	0.260 (0.254)		
	Large	0.501 (0.253)		
	Very large	1.007 (0.262)		
	Male		7.626 (6,5)	0.007
	Small (Intercept)	3.349 (0.299)		
	Medium	−0.029 (0.251)		
	Large	0.156 (0.236)		
	Very large	−0.574 (0.281)		
	Female × Male		7.937 (19,10)	0.541
	Size difference		0.386 (11,10)	0.535
Fertilization probability	Female		0.714 (7,4)	0.870
	Male		2.188 (10,7)	0.534
	Female × Male		14.21 (19,10)	0.115
	Size difference		5.172 (11,9)	0.075
Egg size	Female		2.856 (9,6)	0.414
	Male		6.246 (4,3)	0.012
	Small (Intercept)	0.520 (0.004)		
	Medium	−0.010 (0.006)		
	Large	0.003 (0.005)		
	Very large	−0.006 (0.007)		
	Female × Male		4.063 (18,9)	0.907
	Size difference		0.562 (11,9)	0.755
Egg survival probability	**Female × Male**		20.73 (17,8)	0.014
	Female			
	Small (Intercept)	2.227 (0.685)		
	Medium	1.714 (1.002)		
	Large	−0.805 (0.943)		
	Very large	1.378 (1.046)		
	Male			
	Small (Intercept)	2.227 (0.685)		
	Medium	2.785 (1.559)		
	Large	−0.066 (0.897)		
	Very large	2.145 (1.138)		

Table 1 (continued)

Trait	Variable	Parameter values (SE)	χ^2-value[a] (df)	*P*-value[b]
	Size difference		1.599 (11,9)	0.450
Hatching probability	Female		3.794 (8,5)	0.285
	Male		4.136 (3,2)	0.042
	Small (Intercept)	0.162 (0.449)		
	Medium	0.657 (0.630)		
	Large	0.598 (0.580)		
	Very large	1.631 (0.687)		
	Female × Male		5.999 (17,8)	0.740
	Size difference		1.193 (10,8)	0.551
Larval age-at-hatch	Female		2.049 (9,6)	0.562
	Male		4.594 (5,4)	0.032
	Small (Intercept)	1.599 (0.047)		
	Medium	−0.040 (0.062)		
	Large	−0.112 (0.057)		
	Very large	−0.153 (0.065)		
	Female × Male		11.13 (18,9)	0.267
	Size difference		2.562 (11,9)	0.278
Larval length-at-hatch	Female		0.150 (9,6)	0.985
	Male		7.541 (5,4)	0.006
	Small (Intercept)	3.252 (0.039)		
	Medium	0.025 (0.058)		
	Large	0.123 (0.048)		
	Very large	0.071 (0.052)		
	Female × Male		14.49 (18,9)	0.106
	Size-difference		2.058 (11,9)	0.357
Larval yolk-sac volume	**Female**		4.594 (5,4)	0.032
	Small (Intercept)	0.013 (0.001)		
	Medium	0.003 (0.002)		
	Large	0.003 (0.002)		
	Very large	0.008 (0.002)		
	Male		2.049 (9,6)	0.562
	Female × Male		11.13 (18,9)	0.267
	Size difference		0.391 (11,9)	0.822

[a] χ^2-value from the deletion of the variable from the full model

[b] *P*-values derived from the χ^2 –statistics

The number of eggs produced by zebrafish correlated with female's body size (Fig. 1a, Table 1). Very large females released significantly more eggs compared to large, medium-sized and small females (Table 1). Furthermore, females released on average significantly smaller clutches (number of eggs produced over four spawning days) when mated with very large males (49.2±13.1 eggs over four spawning days) compared to matings with large (72.5±7.98), medium-sized (62.2±7.88) or small (62.2±7.33) males (Fig. 1a, Table 1). Neither the interaction nor the size difference between female and male body size did affect the number of eggs produced (Table 1). In terms of clutch size, 36.9% of

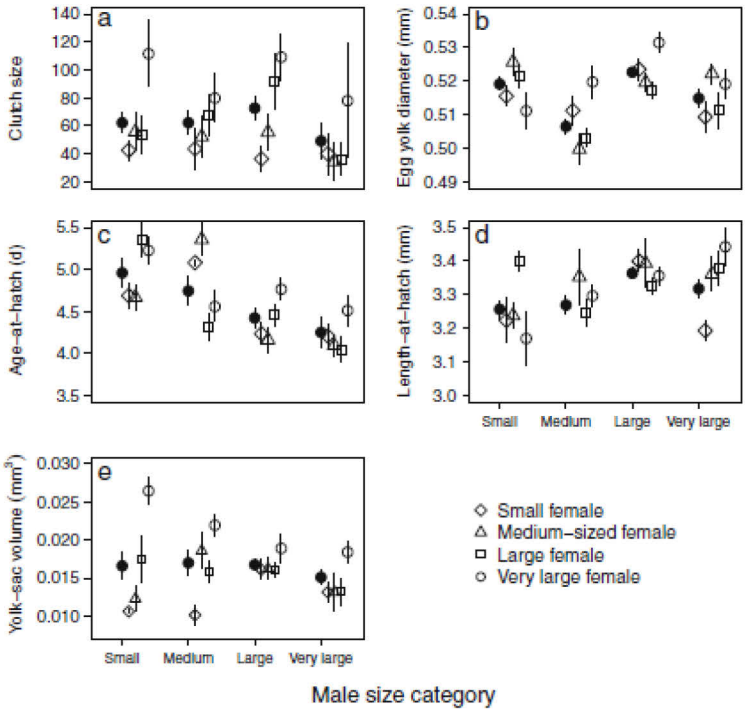

Fig. 1 **a** average clutch size (number of eggs over four spawning days), **b** average egg size, **c** average larval age-at-hatch, **d** average larval length-at-hatch and **e** average yolk-sac volume produced by each male size category across all female size categories (*filled circles*). The average value of crosses between each male and female size category is indicated by the open symbols. *Error bars* indicate standard errors

the variance not captured by parental body size was associated to the individual couples and 7.8% to the spawning day.

The probability of egg fertilization was not influenced by the parental body size or the female × male interaction (Table 1). After controlling for the effect of body size, only 1.2% of the variance was associated to the spawning days, whereas 51.8% of the variance was associated to the individual couples.

Egg traits

We found no difference in egg size (measured as egg yolk diameter) among female size categories and no significant female × male interaction (Table 1). However, females released significantly smaller eggs when crossed with medium-sized males (0.507± 0.002 mm) compared to the eggs released when mated with small (0.519±0.002 mm), large (0.523± 0.001 mm) or very large males (0.515±0.002 mm; Fig. 1b, Table 1). A large proportion of the variance not explained by the parental body size (51.3%) was associated to the individual couples.

In terms of egg survival probability there was a significant interaction between female and male body size, but the pattern was not straightforward (Table 1). Certain combinations, for instance small and large females mated with small males, large males mated with either small or medium-sized females and very large males mated with very large females exhibited lower egg survival probabilities (<90%) compared to other size combinations where egg survival probabilities exceeded 90% (Table 2). 59.8% of the variance in egg survival probability was explained by characteristics other than body size of the individual couple.

Hatching probability was unaffected by female body size, but was affected by male body size. The average hatching probability of embryos fertilized by very large males (0.70±0.02) was significantly higher than embryos fertilized by large (0.61±0.02), medium-sized (0.60±0.02) or small males (0.49±0.02; Table 1). There was no interaction effect between male and female size.

Table 2 The average egg survival probabilities and their standard errors for different female and male size combinations in zebrafish

	Small male	Medium-sized male	Large male	Very large male
Small female	0.827 (±0.03) (*N*=150)	0.989 (±0.01) (*N*=94)	0.889 (±0.03) (*N*=162)	0.979 (±0.01) (*N*=146)
Medium-sized female	0.952 (±0.02) (*N*=188)	0.945 (±0.02) (*N*=201)	0.880 (±0.02) (*N*=251)	0.990 (±0.01) (*N*=105)
Large female	0.760 (±0.04) (*N*=104)	0.956 (±0.02) (*N*=189)	0.955 (±0.01) (*N*=294)	0.963 (±0.03) (*N*=54)
Very large female	0.944 (±0.02) (*N*=124)	0.919 (±0.02) (*N*=136)	0.946 (±0.01) (*N*=350)	0.855 (±0.03) (*N*=173)

N refers to the number of individual eggs used in the egg survival probability estimation

62.1% of the variance in hatching probability was associated to the individual couples.

Larval traits

Regardless of female body size, offspring sired by very large (4.25±0.08 d) and large males (4.42±0.06 d) hatched significantly earlier than offspring of medium-sized (4.74±0.01 d) and small males (4.96±0.01 d; Fig. 1c, Table 1). No variance in hatching time was associated to the individual couples. There was no correlation between the average egg size and the average larval age-at-hatch (df=31, r=−0.127, P=0.483).

Female body size was not a significant variable in determining larval length-at-hatch (Table 1). Instead, larvae which hatched from eggs fertilized by very large (3.32±0.02 mm) and large males (3.36±0.01 mm) exhibited greater standard length than larvae which hatched from eggs fertilized by medium-sized (3.27±0.03 mm) and small males (3.26±0.03; Fig. 1d). When aggregating size categories, very large and large males sired significantly larger offspring compared to offspring of medium-sized and small males (Table 1). In the larval length analysis, 21.5% of the variance, not explained by parental body size, was associated to the individual couples. The average larval length-at-hatch did not correlate with the average egg size (df=31, r=0.180, P=0.316).

Female body size, but not male body size, had a significant effect on larval yolk-sac volume. Very large females produced larvae with significantly greater yolk-sac volume (0.021±0.001 mm^3) relative to large (0.016±0.001 mm^3), medium-sized (0.015±0.001 mm^3) and small females (0.013±0.001 mm^3; Fig. 1e, Table 1). 25.1% of the variance in yolk-sac volume was associated to the individual couples. The average yolk-sac volume did not correlate with the average egg size (df=25, r=−0.031, P=0.880).

Integrative measure of reproductive fitness

The integrative measure of reproductive success (i.e., reproductive fitness) varied with both female and male body size from 0.2 to 4.0 relative to the reproductive value exhibited by small female: small male crossings chosen as the reference category (Fig. 2). Large males, independent of the crossing, had the highest predicted fitness values, which was almost three times higher compared to the reference value of a small female : small male size combination. Particularly the combination of large female and large male yielded the highest reproductive fitness value (4.0), which was four times higher than the reference value. Interestingly, the average absolute reproductive fitness value of very large females was somewhat lower (0.71) compared to the value of large females (1.13), but it was still larger than the average absolute fitness value of medium (0.61) and small females (0.46) across all male sizes. The very large males exhibited the lowest fitness values compared to all other male sizes.

Discussion

Our study is the first to disentangle maternal and paternal-size effects on reproductive success in zebrafish. As expected, female size contributed to reproductive output and larval quality, and more unexpectedly male size contributed to a wide variety of reproductive parameters involving spawning frequency, clutch size, egg size, embryo development rate and larval size-at-hatch. The integrated reproductive fitness measure showed that

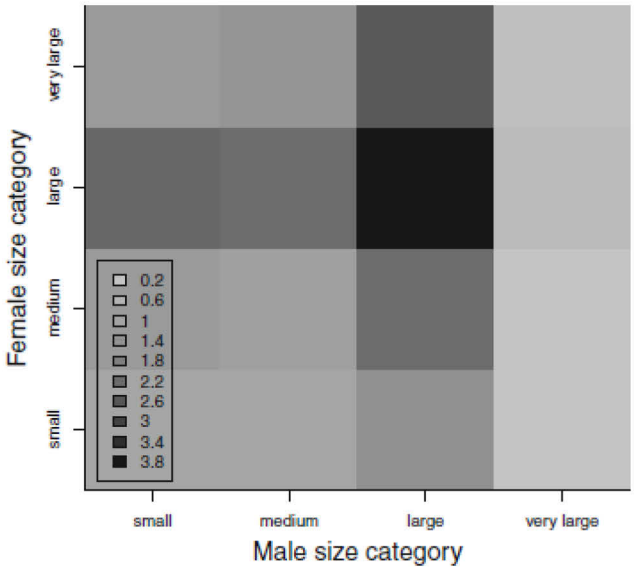

Fig. 2 The estimated integrated reproductive fitness (i.e., the expected number of hatched larvae) for different female and male size combinations in zebrafish. Lighter colour corresponds to lower estimated fitness value. The values are expressed as relative to the reference size category of small female : small male=1. Colors in the figure change smoothly and the example colors in the legend correspond to the relative fitness values

large, but not very large, fish exhibited the highest reproductive success among both males and females, and while large females were reproductively superior to medium and small females, the very large males were the least reproductively fit of all male sizes (Fig. 2). Our results altogether showed that male body size contributes substantially to variation in several early life-history traits in zebrafish, and, therefore, size-dependent paternal effects might be more important for reproductive success in this species than previously believed.

The positive relationship between female body size and fecundity has been shown in several fish species (Wootton 1998) and was also evident in our study, similar to earlier reports in zebrafish (Spence and Smith 2006; Uusi-Heikkilä et al. 2010). In addition to egg number, egg size often correlates positively with female body size across a range of fish species (e.g., Green 2008; Marshall et al. 2008). This is in contrast with our results, which revealed that zebrafish egg size varied independently of female body size. However, it has been previously indicated that egg size measured as egg diameter may not be a biologically relevant parameter for determining zebrafish reproductive success (Uusi-Heikkilä et al. 2010) and this may explain the lack of correlation between egg size and female body size in the present study. The assumption is further supported by the lack of correlation between egg size and a range of larval traits (e.g., length-at-hatch) in our study. In fact, in fish egg quality might be better reflected in embryo developmental rates or larval parameters than in egg size (Kamler 2005, 2008). In our study, very large females did not produce larger eggs, but they produced higher quality larvae, in terms of yolk-sac volume, compared to large, medium-sized or small females. This is consistent with the previous findings of greater egg and larval qualities produced by larger females of many fish species (Marteinsdottir and Steinarsson 1998; Kennedy et al. 2007), as it is known that larvae with larger yolk-sacs may show increased survival in the wild by being more resistant to starvation under food-limited conditions (Miller et al. 1988; Kjørsvik et al. 1990; Marshall et al. 2010). Yet, despite the greater egg numbers and larger larval qualities exhibited by the very large females in our study, they showed a consistently lower integrated reproductive fitness value compared to large females, while still maintaining higher relative fitness compared to medium and small females. This apparently inconsistent finding can be explained by the slightly lower model-predicted spawning probabilities, egg fertilization probabilities and hatching probabilities by eggs produced by very large females compared to large females. The multiplied effects, although

individually not statistically significant (Table 1), surmounted the significantly higher egg number produced by the very large females, resulting in slightly lower reproductive fitness values for very large females. Given the lack of trait-dependent significant differences for female size for many traits such as spawning and fertilization probabilities, one should cautiously interpret this finding and not prematurely discard the reproductive value of very large females.

We identified a range of pronounced paternal-size effects on several reproductive traits such as spawning probability, egg size, clutch size, embryo developmental rate (i.e., hatching time), larval hatching probability, and egg survival probability in zebrafish. Early hatching larvae have higher muscular activity during the embryogenesis compared to late-hatching larvae (Kimmel et al. 1995). Thus, the higher hatching probability and early hatching time of the larvae produced by the large and very large males could be an indicator of faster developmental rate and better larval condition. Previous studies have demonstrated maternal-size effects on embryo developmental rate (Marteinsdottir and Steinarsson 1998; Kennedy et al. 2007), but the evidence for paternal contributions to offspring development is limited (Saillant et al. 2001; Bang et al. 2006). In our study, larvae sired by very large and large males also hatched significantly larger, in terms of standard length, compared to larvae sired by medium-sized and small males. Larger body size at hatch may increase larval fitness in the wild due to the greater mouth gape and higher swimming activity, which allows the larva to predate more efficiently and use wider variety of prey (Miller et al. 1988).

Considering that the contribution of sperm to offspring development is mostly genetic, hypothesizing on the nature of direct, non-genetic paternal effects on early-life history traits is challenging. It has been shown that the amount and quality of sperm varies among males and this variation can be size-dependent (Howard et al. 1998). In zebrafish, however, sperm quality, quantity and motility have been shown to vary with fish age and swimming activity level (Kemadjou Njiwa et al. 2004) but not with body size (Skinner 2004). This is partly supported by our results, which showed no differences in egg fertilization probability explained by male body size. Therefore, we are not convinced that size-dependent sperm quality is a sufficient explanation for the pronounced size-dependent paternal effects we identified.

Male size may also affect reproductive success in a less obvious indirect way through female mate choice and differential allocation patterns (Skinner and Watt 2007b). Differential allocation patterns are particularly likely when the experimental fish are allowed to spawn freely, as in our experiment, and eggs are not striped and fertilized artificially. Females benefit from the allocation of reproductive resources to better quality mates as these partners may provide better genes or more resources to the offspring (Andersson 1994). For example, in zebrafish territorial males are known to be larger (Spence and Smith 2005) and females allocating reproductive resources towards larger, territorial males may benefit from better oviposition sites. Zebrafish females have indeed been shown to prefer (Pyron 2003, but see Hutter et al. 2010) large males, and they have also been found to differentially allocate eggs towards larger males in a second spawning when mated in short sequences with either a large or a small male (Skinner and Watt 2007b). In our study, female zebrafish thus might have allocated higher quality eggs to larger males because the more territorial (i.e., larger) males may exhibit higher reproductive success, as is empirically shown to be true under low density conditions (Spence and Smith 2005; Spence et al. 2006). Such female preferences for large male body size would be revealed as a significant male-size effect in our statistical analysis. In earlier studies the higher reproductive success by larger males has not been consistently evident (Spence and Smith 2005, 2006; Spence et al. 2006). However, differences in study findings on the importance of male size for reproductive success should be viewed in terms of the male size gradient used in the experiments. For example, Spence and Smith (2006) did not find male size to be related to reproductive success in zebrafish while using males ranging between 33.8 and 37.4 mm. In our study, we used males ranging from 24 to 31 mm. Potentially, the larger size gradient of males in our study facilitated the emergence of clear paternal-size effects on reproductive fitness, which may have involved both direct (e.g., genetic quality, sperm quality) and indirect (differential allocation by females) male-size effects.

The relationship between male size and reproductive fitness in our zebrafish study was nonlinear. In

fact, we found that the very large males exhibited consistently lower reproductive fitness compared to large males (Fig. 2). Unlike among females, the very large males exhibited the lowest integrative reproductive fitness value of all male sizes. Interestingly, very large males sired high quality offspring once spawning occurred, but they had substantially lower spawning probabilities and they received significantly smaller clutches compared to the other-sized males. Because this effect was not caused by the relative size difference between females and males, it appears that the advantages of very large body size are traded off against unknown fitness costs of being too large. We can only speculate about the likely mechanisms, but mating-related physiological or behavioral factors (e.g., courtship behavior and sexual harassment; Partridge and Fowler 1990) may play a paramount role. In *Drosophila melanogaster* male body size have been shown to enhance male's mating success but simultaneously to have a detrimental effect on female's fitness leading to a lower egg number received by the large male (Fowler and Partridge 1989; Pitnick and García-González 2002). So far similar mechanisms of sexual conflict have not been demonstrated in fish, however it is possible that our experimental design facilitated continuous sexual harassment of females by very large males, which may have induced substantial stress on females resulting in reduced matings (Morgan et al. 1999; Small 2004). Thus, the persistent and partly aggressive spawning behavior of very large males, which could lead to a high mating success first, may not be advantageous in repeated spawnings and may introduce fitness costs for both females and males. Investigating the potential costs of mating with very large males in zebrafish constitutes an important avenue for further studies on size-dependent sexual conflict in this species.

Our experimental study controlled for density and sex ratio, which both can affect zebrafish reproductive success (Spence and Smith 2005; Spence et al. 2006) and additionally allowed the fish spawn naturally instead of using artificial fertilization. Zebrafish has been suggested to spawn in groups (Spence et al. 2008) but a recent behavioural study showed that wild zebrafish spawn in pairs rather than in groups (Hutter et al. 2010). Therefore, we believe that our experiment allowed us to determine reliably the effect of body size on reproductive success in zebrafish despite the unnatural spawning conditions the fish were exposed to. Our experimental design allowed us to unravel some additional aspects related to zebrafish reproduction. For example, stocking two fish in a spawning box helped us to reveal the relatively high amount of variation in reproductive output and early life-history traits that was associated to individual couples independent of male or female body size (*sensu* Paull et al. 2008). This additional variation in reproductive success could be related to hormonal factors (van den Hurk and Lambert 1983; van den Hurk et al. 1987), genetic incompatibility (Gerlach and Lysiak 2006), or dominance hierarchies (Pritchard 2001). Our study thus suggests that one should expect a strong effect of body size on reproductive performance but additional factors, potentially related to mate choice, are paramount in explaining reproductive success in zebrafish. The great individual variance in reproductive output has implications for experimental design of studies that investigate reproductive success in zebrafish or use reproductive parameters in ecotoxicological studies as large sample sizes are needed to account for the large variability in individual reproductive performance (Paull et al. 2008).

To conclude, our study is the first to unambiguously identify the maternal and paternal-size dependent effects on zebrafish reproductive success. We revealed an insofar overlooked importance of male body size for reproduction in this species and our findings also suggest the importance of body size-dependent sexual conflict and female differential allocation as potential mechanism explaining the pronounced male-size effects. Implicating beyond our laboratory approach and assuming that similar effects exist in other fish species, our findings of paternal-size effects in addition to maternal-size effects are worth being considered when deriving harvest regulations designed to protect exploited stocks. In particular, our results suggest that ignoring the importance of male body size for recruitment of fish might constitute a shortcoming when assessing the impact of size-selective fishing and skewed sex ratio on recruitment dynamics (Langangen et al. 2011).

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Paper III

III

Differential allocation in zebrafish is robust against previous exposure to different-quality males

Submitted

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Differential allocation in zebrafish (*Danio rerio*) is robust against previous exposure to different-quality males

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ABSTRACT

Organisms typically allocate resources to reproduction in response to the costs and benefits of current and future reproductive opportunities. According to differential allocation hypothesis females allocate more resources to high-quality males, whereas the reproductive compensation hypothesis suggests that under constrained mate choice females invest more resources to low-quality, less preferred males. We tested whether zebrafish, a species with a resource free mating system, express male size-dependent differential allocation patterns in monogamous spawning trials and whether mate preference and reproductive allocation pattern by females are affected by previous male-quality experience indicating plasticity in mate choice. Females were conditioned to large, small or random-sized males (control) for 14 days to influence females' expectations of the future mate quality. Female zebrafish showed a clear preference for large males in terms of spawning probability, clutch size, and likely also egg quality. This pattern was largely independent of the conditioning treatment, providing the first

conclusive evidence of differential allocation in this species. However, when females experienced variation in male size (random-sized conditioning treatment) they discriminated less against small males compared to females conditioned to large and small males. This might suggest that differential allocation is of less relevance in nature than revealed in the present laboratory study, despite our finding of a general preference for large males by zebrafish females.

Keywords: *Sexual selection*; *Mate choice*; *Differential allocation; Reproductive compensation; Zebrafish*

1. INTRODUCTION

Sexual selection represents selection for traits that increase an individual's reproductive success. In many mating systems males compete for access to females (i.e., intra-sexual selection), and females in turn select the most attractive male to mate with (i.e., inter-sexual selection; Andersson 1994). Female mate choice is believed to have evolved because it provides females with direct, material benefits (e.g., nutrition, parental care; Heywood 1989, Kirkpatrick 1996) or indirect, genetic benefits (sexy son and good genes –hypotheses; Zahavi 1975, Lande 1981) that collectively should increase female's fitness. Males of many species develop a range of secondary sexual characters and traits, ranging from body ornaments (e.g., kype and adipose fin in Atlantic salmon, *Salmo salar*, Järvi 1990; antlers in red deer, *Cervus elaphus*, Kruuk et al. 2002) to song duration (e.g., in tree frogs, *Hyla versicolor*, Welch et al. 1998) and distinct coloration (e.g., in guppies, *Poecilia reticulata*, Houde and Endler 1990), to signal their attractiveness as mating partners. In species that lack obvious secondary sexual traits, male body size may constitute an important sexually selected character, and large males of some fish species have been shown to provide females with direct fitness benefits, for example better nests (e.g., minnow, *Pimephales promelas*, Hudman and Gotelli 2007) or better offspring care (e.g., smallmouth bass, *Micropterus dolomieui*, Wiegmann and Baylis 1995). In species where material benefits offered by males and male body size correlate positively, females are consequently expected to prefer mating with a large male (e.g. scorpionfly, *Harpobittacus nigriceps*, Thornhill 1983; desert beetle, *Parastizopus armaticeps*, Rasa et al. 1998; minnow, Hudman and Gotelli 2007). However, females may exercise size-dependent mate choice also in the absence of any obvious material resources offered by the

male (i.e., species with a resource free mating system), in which case mating preferences must be a result of indirect, i.e.,. genetic, benefits offered by the male (Zahavi 1975).

Reproduction is energetically costly and usually affects future growth and survival (Bell 1980, Reznick 1985, Roff 1992). Therefore individuals should weigh costs and benefits of investing in reproduction with their current mate against the expected quality and corresponding fitness prospects offered by future mates (Burley 1988). According to the differential allocation (DA) hypothesis females are expected to invest more when they are paired with high-quality males compared to low-quality males, thereby generating a positive relationship between partner quality and reproductive investment (Burley 1988, Sheldon 2000). A theoretical study showed that DA should be an optimal strategy under many environmental conditions unless future mate choice is constrained (Harris and Uller 2009). In nature, an individual usually experiences variation in the attractiveness of potential mates it can breed with over its lifetime, but selection should nevertheless favor individuals that allocate resources towards mates offering greater fitness prospects (Sheldon 2000). Wide-range empirical support for DA has been obtained across many animal species, and it appears that DA influences many systems of mate choice and parental investment (reviewed in Sheldon 2000).

The DA hypothesis has been contradicted by the reproductive compensation (RC) hypothesis, which suggests that rather than increasing investment when paired with a high-quality partner, females should increase investment when paired with a poor-quality male to compensate for the potential negative effects of low male quality on offspring fitness (Gowaty et al. 2007). An allocation pattern consistent with RC has been reported, for example, in pipefish (*Syngnathus typhle*; Goncalves et al. 2010), mallard (*Anas platyrhynchos*; Bluhm and Gowaty 2004), house mice (*Mus musculus*; Drickamer et al. 2000), and in zebra finch (*Taeniopygia guttata*; Bolund et al. 2009). Unlike DA, which in theoretical models was found robust across broad range of ecological conditions, RC is predicted to occur only under restricted conditions and is particularly likely to occur when future fitness prospects and mating opportunities are low (Gowaty et al. 2007, Harris and Uller 2009). The expected future mate quality thus strongly affects the allocation pattern in both DA and RC hypotheses (Burley 1988, Gowaty et al. 2007). When mate choice is constrained and the expected future mate quality is low or sampling costs for a high-quality mate are high, females may express RC (Gowaty et al. 2007). On the other hand, DA is the expected allocation pattern when the quality of the current mate is higher than that of the future mate (Burley et al. 1988). Ideally, females need

information of the present and future mate quality in order to choose the optimal allocation pattern.

Females typically show variability in their mate preference (Jennions and Petrie 1997). Such variation can be based on genetic differences among females (Bakker and Pomiankowski 1995, Bakker 1999) or it might be environmentally induced and reflect phenotypic plasticity (e.g., Milinski and Bakker 1992, Lehtonen 2007, Olofsson et al. 2011). Previous experience (Bakker and Milinski 1991, Breden et al. 1995, Walling et al. 2008) or social learning patterns (Witte and Ryan 2002, Brown and Laland 2003), in particular, may cause experience-dependent plastic changes in female mate preferences over time. If females are forced to sample potential mates sequentially and to compare the present mate with the ones she met previously, she is likely to rate males based on the attractiveness of a previous male, thus exhibit some plasticity in mate choice (Bakker and Milinski 1991, Milinski and Bakker 1992). In sticklebacks (*Gasterosteus aculeatus*), female's internal standards for male quality have indeed been found to be adjustable so that a given male is rated higher by a female when preceded by a less attractive than by an attractive male (previous male effect; Milinski and Bakker 1992). Furthermore, mating preferences can be lost or reversed when the social environment changes. In particular, any social environment consisting of different-sized males, may mediate the expected future mate quality and thus influence female's allocation pattern in species where male size signals high quality (Walling et al. 2008).

Considering the diversity of mating systems in fish, surprisingly few experimental studies have been conducted on the DA hypothesis in this taxon and especially few studies are present in species, which lack parental care or other obvious male-contributed spawning resources (for notable exception, see Skinner and Watt 2007, Evans et al. 2010). Zebrafish (*Danio rerio*) is a batch spawning fish with no parental care (Spence et al. 2008), and although it lacks clear secondary sexual characteristics, zebrafish females have been shown to discriminate particular traits of males during spawning (Turnell et al. 2003, Spence and Smith 2006). Some studies have shown females to prefer large males (Pyron 2003), whereas other studies have reported contradictory findings both in term of association preference (Hutter et al. 2010) and spawning success (Spence and Smith 2006). Watt et al. (2011) found dominant males to sire more offspring than subordinate males. Dominant males have been shown to be larger and more aggressive than subordinate zebrafish males (Paull et al. 2010). Similarly, territorial males have been found to be larger than non-territorial males (Spence and Smith 2005), with

territorial males siring more offspring at low densities (Spence et al. 2006). In their pioneering study on DA in zebrafish in relation to male body size, Skinner and Watt (2007) showed that zebrafish females allocated more eggs to larger males in the second of the two monogamous spawnings when mated with a small or large male in short sequence under conditions of sensing the presence of both male sizes during the mating trials. Because DA was not expressed in the first spawning, the evidence for differential allocation in zebrafish remains weak. However, although zebrafish males do not provide females any parental care or other obvious direct resource benefits, females may still express DA because large males may provide indirect, genetic benefits (Kirkpatrick and Ryan 1991, Andersson 1994, Spence et al. 2006) and signal territoriality (Spence and Smith 2005) leading potentially to access better oviposition sites.

In the present study, we tested 1) whether females express DA in zebrafish which represents an example of a fish species lacking parental care and other obvious male-derived resources and 2) whether the allocation pattern is plastic and can be altered by manipulating the expected future mate quality by conditioning females to various male sizes. We considered DA a general strategy for many iteropareous organisms that face variation in mate quality. However, such preference might be plastic and amendable to change by manipulating the expected future mate quality (i.e. exposure to different male sizes), so that females reared under constrained availability of high quality males may show plasticity in their allocation pattern and express RC rather than DA, as suggested by Gowaty et al. (2007) and Harris and Uller (2009). Our study is among the few to focus on a fish species with a largely resource free mating system where DA is expected to be less pronounced than in mating systems where males of different qualities provide obvious benefits to females (e.g., nest guarding).

2. MATERIALS AND METHODS

(a) Experimental fish and holding conditions

Our experimental fish were the third generation offspring from a wild zebrafish population captured from a river system 70 km west of Coochbihar (West-Bengal, India, 22.56°N, 87.67°E). Fish were raised in glass fiber – polyester tanks (volume 320 l) in a light (14 h light : 10 h dark) and temperature controlled (mean ± s.d. 26.8 ± 0.79 °C) recirculation facility with

an inflow rate of 0.25 ls^{-1}. The stocking density was 0.9 ± 0.2 individuals l^{-1}. Fish were fed *ad libitum* with Artemia nauplii (Inve Aquaculture NV) and commercial flake food (TetraMin, Tetra GmbH; 47 % protein, 10 % fat).

(b) Conditioning females to different-sized males

To study whether manipulating the expected future mate quality changes female mating preferences or allocation pattern of reproductive resources, we conditioned females to social environments, which consisted of different-sized males (random-sized, large and small) for 14 days (table 1). The conditioning time of 14 days was chosen because it has been shown that zebrafish can learn tasks in as few as 10 days and for example display rapid, reliable food conditioning (Williams et al. 2002, Colwill et al. 2005) and alarm reactions (Hall and Suboski 1995). We thus assumed that zebrafish females would also be able to internalize social preferences and male size structure in a conditioning period of 14 days.

It has been suggested in theoretical models that female and male age may mediate the mate preference (Kokko 1997) and allocation patterns (Jennions and Petrie 1997, Harris and Uller 2009). Therefore, the fish used in our experiment were all the same age (150 days post fertilization; dpf). Zebrafish start maturing at age 90 dpf (Schilling 2002) and our test population's females at length of 19 mm (Uusi-Heikkilä et al. 2011), thus we were confident that all the females used in the experiment were mature (standard length mean ± s.d.: 27.7 ± 1.21 mm). Owing to the potentially aggressive, female stress-inducing behavior by the relatively largest and thus most dominant males in zebrafish (Uusi-Heikkilä et al. 2012), the body size of males assigned to the different conditioning treatments was controlled such that males were never larger than females (table 1).

After the females and males were randomly assigned to the different conditioning treatments, the fish were stocked in aquaria (volume: 45 l), which were controlled for light (14 h light : 10 h dark) and temperature (mean ± s.d. 25.6 ± 1.13 °C). The stocking density was 1.1 individuals l^{-1} with the 1:1 sex ratio. In order to block the olfactory cues among the conditioning treatments, fish assigned to different treatments were reared in separate recirculation systems (inflow rate of 0.14 ± 0.43 l s^{-1}), and additionally the aquaria were covered to prevent any visual contact among fish during the conditioning.

(c) Spawning period and data collection

After 14 days of conditioning the females to different-sized males, a two day spawning trial was initiated to study the potential differences in reproductive allocation among females originating from different social environments. To obtain a maximal reproductive output in such a short time period, females were isolated from males for 24 hours (Westerfield 1994) after which the fish were transferred into spawning boxes (volume: 3 l) designed to prevent egg cannibalism by separating the spawning fish from the eggs by a mesh construction (Aquarien-Bau Schwarz, 37081 Göttingen, Germany). Individual females from different conditioning treatments (i.e., reared with random-sized, large or small males) were stocked into the spawning boxes either with a large male (27.2 ± 1.41 mm) or a small male (22.6 ± 1.06 mm). The difference in body length between large and small males was statistically significant ($t_{105.9} = 20.81$, $p < 0.001$). It has been shown that familiarity can influence fish behavior and breeding performance (e.g. Griffiths et al. 2004, Gómez-Laplaza and Fuente 2007), therefore the males used in the spawning trial were novel individuals (i.e. reared separately) to avoid any bias in reproductive performance caused by potential mate familiarity. Large ($t_{87.07} = -0.303$, $p = 0.762$) and small males ($t_{122.7} = 1.337$, $p = 0.184$) used for conditioning and for spawning did not differ in their standard length. From all three conditioning treatments 10 females were coupled with a large male and 10 females were coupled with a small male, except from the random-sized male conditioning treatment only seven females were coupled with a large male. Spawning boxes were stocked with one female and one male and visual and chemical contact was prevented among the boxes during the two days spawning period.

Zebrafish spawn within the first few hours after sunrise (Hisaoka and Firlit 1962), thus the assessment of reproductive output took place between 0800 and 1000 hours (automatic light-on in the spawning facility was at 0600 hours). The spawning boxes were cleaned on both days, the occurrence of spawning was assessed and the number of eggs was counted. For assessing the egg fertilization probability, we enumerated fertilized eggs from unfertilized eggs. Only clutches larger than 20 eggs were used in the egg fertilization probability estimation to avoid inflated egg fertilization probability estimates due to random egg mortalities in very small clutch sizes.

(d) Statistical analyses

We used generalized linear model (GLM) to determine the effect of conditioning treatment and male body size on spawning probability, clutch size (i.e. number of eggs per female per

day) and egg fertilization probability. In all of the analyses, rearing treatment (i.e. random-sized, large or small males), male size (i.e. large or small male) and their interaction were treated as fixed effects. The individual couple and spawning day were set as random effects to estimate the variation among couples and between the days that could not be related to the conditioning treatment or male body size. The amount of variance associated to the random variables was estimated through variance components. Couples which did not produce any eggs during the two-days spawning period were excluded from the clutch-size analysis. Clutch size was modeled through Poisson regression and spawning probability and egg fertilization probability were modeled through binomial regressions. If data were over-dispersed, the quasi-Poisson or quasi-binomial distributions were used. Statistical significance of fixed effects was determined by chi square test comparisons of successively simpler models, which agreed with Akaike Information Criterion (AIC) model selection methods.

In the results, mean values are presented with standard errors. All data were considered statistically significant at $p< 0.05$. All statistical analyses were performed with R 2.13.1. with the lme4 package (R Development Core Team 2009).

3. RESULTS

Male size during spawning trials had a significant effect on spawning probability, while the conditioning treatment did not affect it, and there was no interaction between the conditioning treatment and male size (table 2). The spawning probability was higher when spawning occurred with large (0.46 ± 0.05) relative to small males (0.25 ± 0.04; figure 1a, table 2). A substantial amount of variation (76.3 %) was associated to the individual couples. Spawning day could be excluded from the model since virtually no variation was associated to this variable (< 0.001 %).

The average clutch size (number of eggs produced by a female per day) was significantly higher among females crossed with large males (169 ± 25.1) compared to females crossed with small males (94.9 ± 18.5; figure 1b, table 2) when pooled across the conditioning treatments. The conditioning treatment did not have a significant effect on clutch size, although it approached statistical significance ($P = 0.073$, table 2). Indeed, it appeared that the DA pattern in the random-sized male treatment was less pronounced than in the conditioning

treatment with large and small males. The interaction between conditioning treatment and male size did not have a significant effect on egg production (table 2). In terms of clutch sizes, 48.7 % of the variation was associated to the individual couples and 8.08 % to the spawning days.

Conditioning treatment did not have a significant effect on egg fertilization probability but as in clutch size there was a strong trend ($p = 0.069$) in terms of differences in egg fertilization probability between large and small males: the difference was smaller in the random-sized treatment (large male: 0.63 ± 0.01, small male: 0.69 ± 0.01) compared to females reared with either large (large male: 0.55 ± 0.01, small male: 0.41 ± 0.03) or small males (large male: 0.51 ± 0.01, small male 0.32 ± 0.02; figure 1c). Similarly, the male size effect approached statistical significance ($p = 0.064$) as the egg fertilization probability was higher across the conditioning treatments among females coupled with large males (0.56 ± 0.01) compared to small males (0.47 ± 0.01). The interaction between the rearing treatment and the male size was not significant (table 2). In terms of egg fertilization probability, 56.6 % of the variance was associated to the couples and 1.78 % to the spawning day.

4. DISCUSSION

In accordance with our study hypothesis, we demonstrated that zebrafish females differentially allocate reproductive resources in terms of egg numbers based on the size-dependent attractiveness of their mate and they also show a greater propensity to spawn with larger males. Furthermore, the egg fertilization probability, which given the absence of a correlation between male body size and the amount or the quality of sperm in zebrafish (Skinner 2004) can be interpreted as an indicator of egg quality (e.g., Bromage et al. 1994), was higher in spawning trials involving large males. This suggests that females released more and higher quality eggs to large males compared to smaller conspecifics. We found this result to be largely robust against the male-size dependent social environment females were previously exposed to, although the differential allocation (DA) pattern seemed to be less pronounced in the control treatment where females were exposed to random-sized males prior to spawning. Our findings constitute the first unambiguous demonstration of DA in zebrafish as an example of a resource free mating system. By contrast, we found no evidence for the reproductive compensation (RC) hypothesis (i.e., females allocating more reproductive

resources towards low quality, small males; Gowaty et al. 2007) in zebrafish as the allocation pattern was largely unaffected by the conditioning treatment.

Our study is one of the few studies (see also Reyer et al. 1999, Skinner and Watt 2007, Evans et al. 2010) demonstrating the existence of DA in a species with a resource free mating system, thereby complementing the more contrived finding of DA previously reported in zebrafish by Skinner and Watt (2007). In their study females could sense the presence of an alternative male in all monogamous spawning trials, even when females were temporarily exposed to a less preferred male. Therefore, the females may have released eggs for a less preferred male (i.e. small) in a first spawning as spawning with a more preferred male (i.e. large) was perceived possible in the future. In our study, on the other hand, females were isolated visually and chemically from other study males and unlike Skinner and Watt (2007), we were able to immediately show a clear pattern of DA. Although females conditioned to large males (28.2 mm) were on average slightly larger compared to females conditioned to small (27.6 mm) and random-sized males (27.2 mm, table 1), our results seemed to be unaffected by that as females conditioned to large males expressed a similar level of DA relative to females conditioned to small males (figure 1).

We found that the DA by female zebrafish was less pronounced among females held with random-sized males where the conditioning treatment consisted of both small and large males. These females appeared to be less discriminative towards small males potentially because the continuous variation in male size did not promote perceptual discrimination against unpreferred (Pyron 2003), small males as females were trained to expect the possibility to encounter a large male in the future. Females reared with small males expressed clear DA pattern as their spawning probability and egg allocation was greater when the quality of the male encountered was higher (large male) than the expected future mate quality (small male). Females conditioned to large males, on the other hand, may have developed a threshold criterion (Wittenberger 1983) according to which they do not mate or at least allocate less reproductive resources to males not exceeding the threshold (small male). Obviously we can only speculate about the plausible explanations regarding the previous male effect (Bakker and Milinski 1991) but the fact that females reared with random-sized males seemed to be less discriminative in terms of allocating reproductive resources (i.e., clutch size and egg quality) suggests some degree of socially mediated plasticity in allocation, which possibly reduces the prevalence of DA in more natural conditions.

In various species, females can enhance their fitness by choosing a mate who can provide, for example, a better nest site or more intensive care for the offspring (Thornhill 1983, Price 1984, Lightbody and Weatherhead 1988). The mechanism for why female zebrafish prefer (Pyron 2003) and strategically allocate eggs to larger males is less clear as this species lacks obvious male-derived spawning resources. However, it has been shown that in zebrafish male body size correlates with territoriality and it is known that females release more eggs to territorial males compared to non-territorial ones (Spence and Smith 2005). Territorial males might provide females better oviposition sites, thus an important resource for the developing larva, and this might be the mechanism why females prefer spawning with larger males. However, in semi-natural conditions males rarely express territoriality but mostly pursue females actively (Hutter et al. 2010). Thus, it is not clear whether territoriality is a typical behavioral strategy among zebrafish males in nature. In addition to direct benefits, zebrafish females may also receive indirect, genetic benefits from large males. According to the good genes hypothesis females gain an evolutionary advantage by mating with a high-quality male and passing those genes on to the offspring (Zahavi 1975). In zebrafish, large male body size can be associated with high male quality as large individuals are more dominant in the social hierarchy and thus are able to govern the feeding opportunities (Grant and Kramer 1992, Hamilton and Dill 2002, Filby et al. 2010). Indeed, large (but not very large) males have been previously reported to exhibit higher reproductive success compared to small males (Spence and Smith 2005, Uusi-Heikkilä et al. 2012). However, it has not been shown whether the mechanisms behind the high reproductive success of large males were direct paternal effects or indirect maternal effects related to female differential allocation. Results of our study clearly suggest that female DA may play a prominent role in determining the higher reproductive success of large males. One has to keep in mind, though, that the higher spawning probability and per capita egg production to large males in the present study may have been facilitated by male behavior, not female allocation. Large males may be more active and aggressive in initiating spawning than small males and this could have lead to higher spawning probability, egg production and fertilization rate (Foote et al. 1997, McGhee et al. 2007). In zebrafish, however, the very small subordinate males have been shown to sire more offspring compared to larger-sized subordinates in the presence of dominant males potentially to compensate for their overall lower attractiveness (Watt et al. 2011). Because we found clear evidence for discrimination against the small males, male behavior is not a plausible mechanism explaining the differential egg production observed in our study. We are

thus confident that we have documented female mate choice and DA rather than male ability to stimulate female egg release.

There is evidence that social factors are important in influencing mate choice and that experience with different male phenotypes can affect mate preferences in fish (Breden et al. 1995, Verzijden and ten Cate 2007, Walling et al. 2008). Therefore, it was reasonable to assume that in zebrafish the preference and allocation pattern may also be adjusted, at least to certain degree, by changing the social environment. We predicted that low frequency of high quality-males (i.e., females conditioned to small males) leads to increased sampling costs among females and shift their preferences so that less attractive males are also accepted (Bakker and Milinski 1991, Milinski and Bakker 1992). However, in contrast to our expectations changes in social environment did not induce plasticity in female mate preference or strongly alter the egg allocation pattern. Instead females consistently preferred large males independent of the conditioning treatment, although there was a trend for a weakened discrimination against small males in our control group. It has been reported that strong social preferences are formed when zebrafish are juveniles (Engeszer et al. 2004, Moretz et al. 2006), and conditioning mature adults for a relatively short time period was potentially not enough to create a strong social imprinting. Although short term changes in adult social environment might not induce a plastic response in zebrafish mate preference and allocation pattern, changes in density and sex-ratio might alter the reproductive success of different-sized males. It has been shown that large (territorial) males have higher reproductive success, potentially reflecting female DA, in low densities (3 individual groups), but not in high densities (15 individual groups; Spence and Smith 2005, Spence et al. 2006). In high densities the female oviposition may be interrupted more frequently and the risk of egg cannibalism may be enhanced (Spence and Smith 2006), thus high density may introduce relatively high fitness costs to the female. Consequently, females may reduce their selectivity and allocate equal amounts of reproductive resources to small and large males (Spence and Smith 2005). Alternatively, this result could have been a consequence of intense male-male competition where male interactions played a predominant role and overwhelmed female preferences. We admit that the results from isolated monogamous studies, such as ours, may not always be accurate indicators of mate preferences and differential allocation in nature. In fact, it is possible that female mate choice and allocation can be altered by the presence of other males and females, thus more realistic social interactions (McGhee et al. 2007). However, it has been shown that wild zebrafish spawn in pairs rather than in groups (Hutter et

al. 2010) and therefore the allocation pattern is potentially less affected by density-dependent interactions among individuals in nature. Further studies are needed to investigate the effect of density and group size in zebrafish spawning behavior and reproductive success in natural conditions.

We showed that females prefer and allocate more eggs towards large males compared to small ones. Although large zebrafish males may be preferred by females and superior in many ways compared to small ones, large males may have a selective disadvantage under specific environmental conditions (Kasumovic and Andrade 2009, Watt et al. 2011), which were not part of our experimental design. As competitive environments fluctuate, there may be no single optimal phenotype, and to maximize fitness, individuals must match their phenotype to the specific competitive challenge they are likely to encounter (Lehtonen 2007, Kasumovic and Andrade 2009). Although zebrafish females preferred large males over small ones, some females spawned with and produced eggs to small males as well. This behavior was particularly pronounced among females reared with random-sized males, an environment that reflects the variation in male body size occurring in nature. In zebrafish, small males are suggested to actively pursue females whereas large males guard territories (Spence and Smith 2005, but see Hutter et al. 2010) and it is possible that owing to these different male reproductive behavior small males have a selective advantage under specific competitive contexts (Watt et al. 2011), thus selection for male body size is not expected to be directional. We nevertheless showed for the first time in zebrafish that female preference towards large males was relatively persistent across different social environments, potentially reflecting high fitness benefits females can receive from large males in benign, laboratory environment. However, a future goal could be to broaden the search for the forces causing female mate preference and to study how these mechanisms interact. It would be important, in particular, to know what environmental variables (e.g., predation risk, competition, social interactions) can induce variation in mate preference and egg allocation, how repeatable the results are at the individual level and how the variation in female preferences affects the mean offspring fitness.

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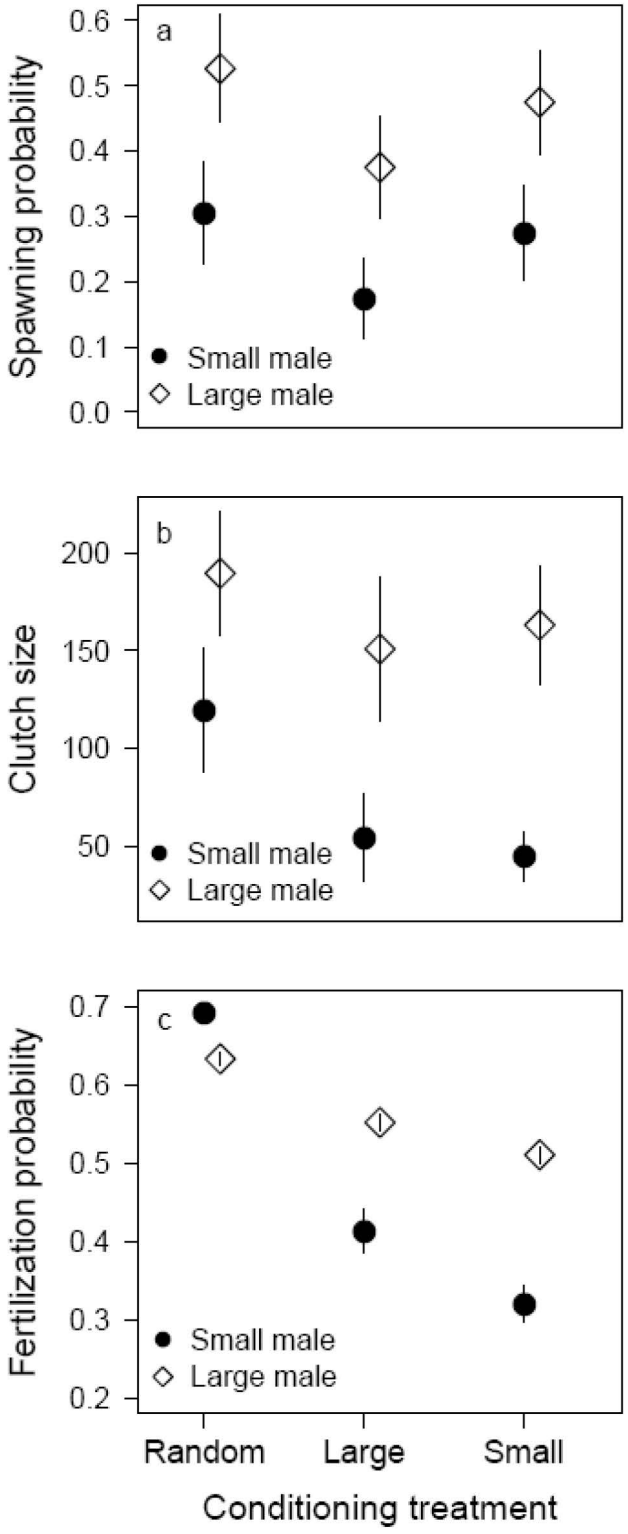

Figure 1. a) average spawning probability, b) average clutch size per day, and c) average egg fertilization probability among females from different conditioning treatments coupled with either large or small males. Error bars indicate standard errors.

Table 1. The standard length (mean ± sd) of females and males used in different conditioning treatments. N indicates the number of females (i.e. the number of individual couples) used in the spawning trial.

Conditioning treatment	Females	Males
Random-sized males	27.2 ± 0.69 mm	25.0 ± 2.49 mm
	N = 37	
Large males	28.2 ± 1.45 mm	27.1 ± 1.54 mm
	N = 40	
Small males	27.6 ± 1.17 mm	22.7 ± 1.31 mm
	N = 40	

Table 2. The effect of conditioning treatment, male size and their interaction on reproductive parameters in zebrafish. Significant predictors are indicated in bold.

Trait	Variable	Estimated parameter values (SE)	χ^2-value[a] (df)	*P*-value[b]
Spawning probability	Treatment		3.052 (5,3)	0.217
	Male size		9.123 (3,2)	0.003
	Small (Intercept)	-1.737 (0.350)		
	Large	1.465 (0.467)		
	Treatment × Male size		0.011 (7,5)	0.994
Clutch size	Treatment		5.246 (7,5)	0.073
	Male size		11.34 (5,4)	0.001
	Small (Intercept)	3.633 (11.28)		
	Large	1.162 (8.985)		
	Treatment × Male size		1.640 (9,7)	0.440
Fertilization probability	Treatment		5.342 (7,5)	0.069
	Male size		3.419 (6,5)	0.064
	Treatment × Male size		1.340 (9,7)	0.512

[a] χ^2 -value from the deletion of the variable from the full model

[b] P-values derived from the χ^2 –statistics

Paper IV

IV

Size-dependent reproductive success of wild zebrafish *Danio rerio* in the laboratory

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Size-dependent reproductive success of wild zebrafish *Danio rerio* in the laboratory

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Size-dependent reproductive success of wild zebrafish *Danio rerio* was studied under controlled conditions in the laboratory to further understand the influence of spawner body size on reproductive output and egg and larval traits. Three different spawner size categories attained by size-selective harvesting of the F_1-offspring of wild *D. rerio* were established and their reproductive performance compared during a 5 day period. As to be expected, large females spawned more frequently and had significantly greater clutch sizes than small females. Contrary to expectations, small females produced larger eggs when measured as egg diameter with similar amounts of yolk compared to eggs spawned by large spawners. Eggs from small fish, however, suffered from higher egg mortality than the eggs of large individuals. Embryos from small-sized spawners also hatched later than offspring from eggs laid by large females. Larval standard length (L_S)-at-hatch did not differ between the size categories, but the offspring of the large fish had significantly larger area-at-hatch and greater yolk-sac volume indicating better condition. Offspring growth rates were generally similar between offspring from all size categories, but they were significantly higher for offspring spawned by small females in terms of L_S between days 60 and 90 post-fertilization. Despite temporarily higher growth rates among the small fish offspring, the smaller energy reserves at hatching translated into lower condition later in ontogeny. It appeared that the influence of spawner body size on egg and larval traits was relatively pronounced early in development and seemed to remain in terms of condition, but not in growth, after the onset of exogenous feeding. Further studies are needed to explore the mechanisms behind the differences in offspring quality between large- and small-sized spawners by disentangling size-dependent maternal and paternal effects on reproductive variables in *D. rerio*.

Key words: egg size; offspring growth; parental effects; reproductive output; size-at-hatch.

INTRODUCTION

Body size is a fundamental trait affecting many aspects of fish performance, including individual reproductive output (Peters, 1983; Marshall *et al.*, 1998; Marteinsdottir & Begg, 2002; Persson & de Roos, 2007; Sogard *et al.*, 2008). For example, in many

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fish species, an allometric relationship between female body size and fecundity exists resulting in fecundity scaling linearly with body mass (Wootton, 1998). Additionally, in some species male body size and condition are important during competition for access to females during spawning (Fleming & Gross, 1994; Quinn & Foote, 1994). Small fishes may also face energetic constraints and low condition, which may result in delay, or even skipping, of reproduction (Rideout *et al.*, 2005) to increase future reproductive output and fitness by growing to a larger size (Houston & McNamara, 1999; Jørgensen & Fiksen, 2006). Alternatively, to compensate for reduced fecundity, small individuals may invest in gamete quality by increasing the size of gametes (Smith & Fretwell, 1974; Hendry *et al.*, 2001) or the amount of resources allocated to gametes (Murry *et al.*, 2008). These compensatory investments into gamete quality, however, have received little attention as much research has concentrated on studying positively size-dependent parental effects (*i.e.* parents' non-genetic contribution to offspring's phenotype; Bernardo, 1996) on reproductive variables. This literature usually reports that large spawners produce higher quality eggs and larvae than small spawners, which in turn can be more resistant to cope with various environmental challenges (Blaxter, 1986; Chambers & Leggett, 1996; Berkeley *et al.*, 2004; Gregersen *et al.*, 2009). These parental effects have been found to be pronounced early in development and to decay rapidly after the larval phase (Bernardo, 1996; Heath & Blouw, 1998). Limited field studies, however, are available on the long-term parental effects on offspring quality and performance (Heath & Blouw, 1998), presumably due to the difficulties in tracking individual reproductive success in field settings.

To improve basic understanding of the effects of spawner body size on reproductive traits such as egg production, egg traits and offspring characteristics, experimental studies with small-bodied model species conducted under controlled conditions are advisable. One of the most popular model species in developmental biology is the freshwater cyprinid zebrafish *Danio rerio* (Hamilton). Despite its small size and capability to produce eggs frequently, this species has only occasionally been used to investigate size-dependent reproductive variables (Skinner, 2004; Spence & Smith, 2006). Moreover, available studies concerning *D. rerio* egg production and reproductive success have used domesticated strains well habituated to laboratory conditions, and limited knowledge is available on the size-dependent reproductive success of wild *D. rerio*.

Danio rerio is a small-bodied species native to India, Bangladesh and Nepal (Spence *et al.*, 2008). Natural spawning is seasonal and reproduction may be cued by food availability (Spence *et al.*, 2006) and quality (Meinelt *et al.*, 1999). As a batch-spawner, *D. rerio* females spawn relatively large eggs in small clutches over a short period at dawn (Spence *et al.*, 2007). Domesticated strains spawn continuously at intervals of 1 to 6 days and clutch size is known to correlate positively with both female body size and inter-spawning interval (Spence & Smith, 2006). There is also some evidence that female reproductive success may be correlated with male size as females prefer (Pyron, 2003) and strategically allocate their eggs towards larger males if exposed to both small- and large-sized males in sequence (Skinner & Watt, 2007). Other studies, however, have shown that the female reproductive success in *D. rerio* does not correlate with male body size (Spence & Smith, 2006) but with other, yet unresolved, male characteristics, and olfactory cues or genetic compatibility may play a decisive role (Gerlach & Lysiak, 2006).

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In addition to mate preferences, sex ratio and population density may have an effect on *D. rerio* mating behaviour and mating success (Spence & Smith, 2005). High density and male- or female-biased sex ratio may lead to increased levels of aggressive interactions, which can have a negative effect on the total number of eggs spawned per female (Pritchard, 2001; Spence & Smith, 2005; Paull *et al.*, 2008). Social systems in *D. rerio* are characterized by the establishment of dominance hierarchies, and dominant individuals generally behave aggressively towards the subordinate ones governing access to food resources (Pritchard, 2001). In addition to reproductive success, this may translate into differences in body size and condition between dominant and subordinate individuals, but these relationships are speculative and have not been studied so far.

The objective of the present study was to compare the reproductive performance of differently sized wild *D. rerio* under controlled laboratory conditions. Three different size categories were established by selecting spawners from large laboratory-held wild *D. rerio* populations, and these size-matched spawner populations were compared in terms of their reproductive success, egg and larval traits and offspring growth. It was hypothesized that 1) large fish would perform better in terms of egg output than small fish, 2) large fish would produce larger and better conditioned egg and larvae than small fish and 3) the offspring of the large fish would develop earlier and grow faster in consecutive months than offspring from small fish due to provisioning effects associated with parental body size on egg and larval quality.

MATERIALS AND METHODS

FISH AND HOLDING CONDITIONS

The fish used in this experiment were first-generation offspring from a wild *D. rerio* population captured from a river system 70 km west of Coochbihar (West Bengal, India, 22°56′ N; 87°67′ E) and reared under laboratory conditions for 6 months. Fish were raised in six round, grey tanks (material: glass fibre–polyester, diameter: 79 cm, height: 135 cm, volume: 320 l) in a light (14L:10D) and temperature-controlled (mean ± S.D. 25·3 ± 0·2° C) recirculation facility with an inflow rate of 0·25 l s^{-1}. The recirculation system was run with insipid tap water, and the water quality was controlled weekly for pH (mean ± S.D. 8·4 ± 0·1), nitrite ($N\text{-}NO_2^-$; <0·3 mg l^{-1}) and ammonium ($N\text{-}NH_4^+$; <0·05 mg l^{-1}) and daily for oxygen levels (mean ± S.D. 8·3 ± 0·3 mg l^{-1}). The stocking density per holding tank was 0·9 ± 0·2 individuals l^{-1} (mean ± S.D.). All fish were reared in identical densities to control for density effects and to minimize the potential bias of dominance hierarchies on feeding success. Fish were fed *ad libitum* five times a day with freshly hatched *Artemia* sp. nauplii (Inve Aquaculture NV; www.inve.com) and a commercial flake food (TetraMin, Tetra GmbH; www.tetra.net; 47·0% protein, 10·0% fat). Multiple feedings of small amounts of food allow fishes to better utilize the food and also spread the food more equally among individuals reducing the size heterogeneity within a tank (Steffens, 1985). Due to the similar stocking densities and feeding rates among the tanks, the potential of social hierarchies differentially influencing the stocks in the six tanks was minimized.

Danio rerio start reaching maturation at *c.* 90 days post-fertilization (dpf; Schilling, 2002), and once all experimental fish had certainly matured at 180 dpf, spawners were selected based on their body length. Fish were caught using a dip-net, and standard length (L_S) was measured to the nearest mm and wet mass (M_W) to the nearest 0·1 g. In addition to L_S and M_W, the Fulton's condition factor (K; Ricker, 1975) was estimated as a proxy for the nutritional state of the three size-matched treatment groups. In two of the tanks, 25% of the largest fish were retained. Fish from these two tanks were pooled into a spawning stock constituting

large males and females (L_S range, 28–35 mm; $n = 191$). In two other tanks, 25% of the smallest fish were retained. Fish selected from these two tanks formed a spawning stock with small-sized spawners (L_S range, 15–24 mm; $n = 162$). Two additional tanks served as controls and were randomly harvested for fish body size. These fish were used as a random-sized spawner treatment (L_S range, 18–35 mm; $n = 210$). The mean ± S.D. K values between large- (0·50 ± 0·08), random- (0·52 ± 0·09) and small-sized spawners (0·52 ± 0·18) did not differ significantly (nested ANOVA, d.f. = 5 and 539, $P > 0·05$).

The reproductive performance of the three size categories (large, random and small) was compared under a controlled laboratory environment that exposed all individuals to similar conditions and densities in terms of number of fish per volume of water, water quality, illumination and type of tank. Before the experiment, males and females from each of the three size categories were kept together within their respective size treatment for 14 days in six (fish selected from one tank per aquarium) 126 l glass aquaria (length: 60 cm, width: 60 cm, height: 35 cm). By the time the experiment was initiated, spawners (*i.e.* spawning females and males) were transferred into a standalone spawning facility (Aquarien-Bau Schwarz; www.aquaschwarz.com; mean ± S.D. temperature: 26·1 ± 0·1° C; mean ± S.D. pH 8·4 ± 0·1; $N\text{-}NO_2^-$; <0·3 mg l^{-1}; $N\text{-}NH_4^+$; <0·05 mg l^{-1}; mean ± S.D. oxygen level 8·3 ± 0·4 mg l^{-1}) in spawning boxes specifically designed to prevent egg cannibalism by separating adults from eggs. This was achieved by inserting a grid with a mesh-size of 2 × 2 mm inside each spawning box. Each spawning box was equipped with green plastic filter material serving as a spawning substratum. Fish were allowed to spawn in two differently sized boxes: spawning boxes of 5 l volume (length: 29 cm, width: 12 cm, height: 10 cm) were stocked with two females and four males and 3 l boxes (length: 21 cm, width: 11 cm, height: 13 cm) were stocked with one female and two males. Preliminary trials testing the effect of box size (with twenty-two 5 l and twelve 3 l boxes) on reproductive metrics revealed no significant effects of box type on reproductive variables (spawning frequency: two-way ANOVA, d.f. = 1 and 32, $P > 0·05$; clutch size: repeated-measures ANOVA, d.f. = 1 and 28, $P > 0·05$; fertilization rate: mixed-model GLM, d.f. = 1 and 54, $P > 0·05$), thus the data from differently sized boxes were pooled to compare the reproductive performance of the three size categories. All female-related data were expressed on a per-female basis by dividing the spawning frequency and the daily egg number with the number of females per box following Paull *et al.* (2008). Due to limited number of boxes for spawning trials and slightly different number of spawners in each size category (due to unequal sex ratios), the final number of boxes used varied among the large ($n = 34$), small ($n = 54$) and random size categories ($n = 25$). Each box of each size category was treated as a replicate. The reproductive output of the fish from the three different size categories was assessed for five consecutive days (29 November to 3 December 2007). Domesticated zebrafish are known to spawn every 1 to 6 days (Spence & Smith, 2006), thus a 5 day spawning period was expected to allow each fish to spawn at least once. Five days was expected to be sufficient time to establish the spawning pattern because *D. rerio* have been shown to spawn for 20 consecutive days without significant changes in egg output as a function of increasing study time (Paull *et al.*, 2008).

REPRODUCTIVE PERFORMANCE

Reproductive output

Danio rerio spawn within the first few hours after sunrise (Hisaoka & Firlit, 1962), and all trait measurements took place between 0800 and 1200 hours to ensure complete spawning and assessment of egg production. To estimate reproductive performance, daily spawning frequency (estimated through identification of eggs spawned) and clutch sizes (*i.e.* the number of eggs spawned per female per spawning event) were assessed for every box over the 5 day spawning period. Accordingly, each morning the spawning boxes were cleaned, the occurrence of a spawning event (*i.e.* spawning frequency) assessed and the total number of eggs counted. For assessing the fertilization rate per spawning event, fertilized eggs were separated from unfertilized eggs. The eggs are translucent and fertilized eggs can be identified by the presence of a multi-cellular blastodisc, which is not present in unfertilized eggs (Kimmel *et al.*, 1995).

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Egg traits

Egg trait measurements for the three size classes included the assessment of egg mortality rate, egg diameter and egg yolk diameter. To estimate post-fertilization egg mortality rate in each of the five spawning days, a pooled sample of 50 fertilized eggs from each of the size categories were placed in five Petri dishes, but only for spawning events with at least $n = 50$ eggs. Petri dishes were transferred into a rearing incubator (Tintometer GmbH; www.tintometer.de) at 27° C. Embryos are known to develop faster in slightly higher temperatures than the 26° C spawning temperature (Westerfield, 1994) and because *D. rerio* embryos are not particularly sensitive to small differences in temperature (Westerfield, 1994), eggs were incubated in a temperature of one degree higher than the spawning temperature. The egg mortality rate for each size category was estimated by counting the number of dead eggs 48 h post-fertilization from each of the Petri dishes. The egg size was measured both as egg diameter and as egg yolk diameter from randomly selected and pooled eggs within size categories. Yolk is the main energy component of an egg, thus egg yolk diameter is relevant in indicating the nutrient resources supplied to an embryo (Kamler, 2005). The eggs were photographed and the diameter was measured from these photographs under a profile projector with an accuracy of 0·0001 mm (Quick Scope; AT112-220F; www.mitutoyo.co.jp). The egg diameter measurements were conducted for three spawning days (the first, second and the fifth spawning day). Yolk diameter was measured from eggs at the gastrulation stage. Because not all the eggs from every spawning day were in this developmental stage by the time they were photographed, measuring the same eggs for egg diameter and egg yolk diameter was not possible. Thus, the egg yolk diameter measurements included eggs from three different days.

Larval traits

Larval traits for the three size categories were assessed as larval hatching time, larval length-at-hatch, larval area and larval yolk-sac volume. Larval trait measurements were treated as pooled samples for the three size categories. Larvae hatched in the rearing incubator in several Petri dishes. The L_S of a random sample of newly hatched larvae from each size category (large: $n = 63$, random: $n = 72$, small: $n = 45$) was measured under the dissection scope, and the hatching time was recorded. The same aged larvae (4 dpf) from three spawning days were used to compare the larval L_S-at-hatch between the size categories. Measurements based on larval length, however, may not be a reliable size-related morphological indicator due to variability in body shape (Kamler, 2008). Therefore, individual larvae were photographed to allow the subsequent measurements of larval area and yolk-sac volume as a more precise indicator of the dimension of the larvae (Heyer *et al.*, 2001; Probst *et al.*, 2006). Larval area and height and width of the yolk sac were measured from photographs using the digitizing software Image Tool for Windows (version 3.0; http://ddsdx.uthscsa.edu/dig/itdesc.html). The yolk-sac volume (V) was then estimated using the following formula (Chambers *et al.*, 1989): $V = \pi(6LH^2)^{-1}$, where L represents the length (horizontal measurement; mm) and H the height (vertical measurement; mm) of the yolk sac. The yolk-sac volume and larval area-at-hatch were estimated over 3 days.

Offspring growth

Two days after hatching, all larvae were transferred into 5 l plastic boxes in the standalone unit and external feeding was initiated. Larvae from each size category were reared separately in replicate boxes in which density varied from 20 to 30 individuals l^{-1}. Larval densities in the boxes were kept well below recommended levels (60 individuals l^{-1}; Brand *et al.*, 2002) to avoid too high densities and stressing the larvae by transferring them into larger rearing boxes after they would have reached larger body sizes surpassing the limits of biomass per box. Fish were fed eight times per day with larval dry food (Sera micron Staubfutter; 50·2% protein, 8·1% fat; www.sera.de) and hatched *Artemia* sp. nauplii. The water inflow rate to the larval boxes was 3 ml s^{-1}, and the photoperiod was 14L:10D. At age 30 days, $n = 10$ fish from 10 rearing boxes per size class were photographed and measured for L_S (mm) and M_W (g). Thereafter, the density was standardized and the fish were further reared in equal

densities (two individuals l^{-1}) in 10 rearing boxes per size category. Body size measurements for a sub-sample of 50 fish per size category were repeated at ages 60 and 90 dpf, and to determine growth rates, growth of individual offspring was related to the mean L_S and M_W of the previous time period. In addition to L_S and M_W, the K of the offspring was estimated at 90 dpf.

STATISTICAL ANALYSIS

The data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene's test). In cases of significant ($P < 0{\cdot}05$) non-normality and heteroscedasticity, data were ln-transformed before parametric analysis and appropriate *post hoc* tests were used that accounted for the nature of the between-sample variance. Logistic regression was used to estimate the probability of spawning with size category as a categorical variable and spawning day as covariate. Repeated-measures ANOVA were used to estimate the effect of the spawner body size on clutch size. Size category was treated as fixed effect and spawning day as a repeated variable. Due to unequal sample sizes, a mixed-model GLM was used to compare egg numbers from successful spawnings and fertilization rates between size classes. Size category was treated as fixed effect and spawning day as repeated variable. The coefficient of variation (C.V.) was calculated in daily egg production and egg size, and one-way ANOVA was used to compare the C.V. of these traits between the size categories. Egg mortality rate, egg size, hatching time and larval traits were analysed using repeated-measures ANOVA, with size category as fixed effect and spawning day as repeated variable. Additionally, the Petri dishes where the larvae hatched were treated as a random effect in repeated-measures ANOVA of hatching time, larval length-at-hatch, larval area-at-hatch and yolk-sac volume, with egg size as a covariate. To analyse differences in offspring body size and K at age 90 dpf, a two-way ANOVA was used with size category treated as fixed effect and rearing box as a random effect. To estimate growth rate in two rearing periods (30–60 and 60–90 dpf), a mean value per rearing box was calculated, and the growth rates were compared between the size categories using one-way ANOVA. All analyses were followed by Sidak's multiple comparison *post hoc* tests or alternatively Dunnett-T3 if variances were still heterogeneous after transformation. In the results, mean values are presented with standard errors (S.E.). All data were considered statistically significant at $P < 0{\cdot}05$. All statistical analyses were performed with SPSS for Windows (Release 14.0.1, SPSS Inc.; www.spss.com).

RESULTS

REPRODUCTIVE OUTPUT

Large- and random-sized fish established a fairly regular spawning activity during the 5 day experiment, while small fish spawned more irregularly. Accordingly, the spawning frequency was more variable among small spawners (C.V. = 33·7%) compared to large (19·5%) and random (21·3%) fish. Over the 5 day spawning period, spawning occurred in 82·4 and 76·0% of all large and random fish spawning trials, respectively, but only in 37·0% of the spawning trials of the small fish. On average, each large ($1{\cdot}0 \pm 0{\cdot}1$) and random female ($1{\cdot}1 \pm 0{\cdot}2$) spawned at least once per 5 days, whereas each small female spawned only $0{\cdot}7 \pm 0{\cdot}2$ times per 5 days. Thus, the inter-spawning interval was on average 5 days for large and random sized and 7 days for small females. Logistic regression analysis revealed a significantly higher likelihood to spawn for large (logistic binary regression, $\beta = 4{\cdot}643$, d.f. = 1, $P < 0{\cdot}001$) and random-sized females ($\beta = 3{\cdot}595$, d.f. = 1, $P < 0{\cdot}001$) compared to small females.

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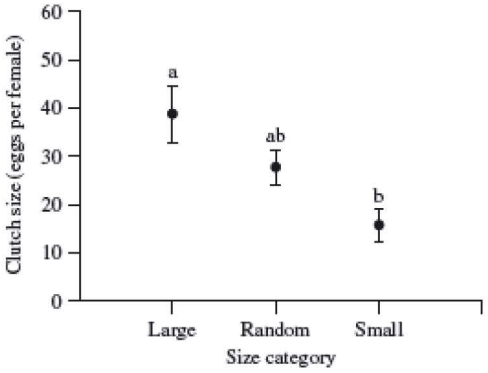

FIG. 1. The effect of *Danio rerio* body size on mean ± S.E. clutch size (repeated-measures ANOVA, d.f. = 2 and 110, $P < 0{\cdot}01$). Large fish: $n = 34$, random-sized fish: $n = 25$ and small fish: $n = 54$. Different lowercase letters indicate significant differences.

The number of eggs produced by wild *D. rerio* correlated significantly with the spawner's body size (repeated-measures ANOVA, d.f. = 2 and 110, $P < 0{\cdot}01$). The mean ± S.D. clutch size (normalized on a per-female basis) per day was higher among large fish (38·7 ± 6·0 eggs per day) compared to small (15·7 ± 3·5 eggs per day), while random-sized fish spawned intermediate clutch sizes (30·0 ± 3·7 eggs per day; Fig. 1). The mean clutch size per day did not vary significantly between the spawning days (d.f. = 4 and 107, $P > 0{\cdot}05$), and there was no size group × spawning day interaction (d.f. = 8 and 214, $P > 0{\cdot}05$). When comparing the number of eggs released in single successful spawnings instead of the average daily clutch sizes, small females (111·6 ± 18·2) produced the same number of eggs per successful spawning as large females (104·5 ± 12·3), and the random-sized females produced slightly fewer eggs (64·6 ± 5·5). These differences were not statistically significant (mixed-model GLM, d.f. = 2 and 69, $P > 0{\cdot}05$). The variance in daily egg production was significantly higher among small (C.V. = 238·8%) compared to random-sized fish (151·5%; one-way ANOVA, d.f. = 2 and 110, $P < 0{\cdot}01$), but there was no significant difference between small and large fish (189·5%; d.f. = 2 and 110, $P > 0{\cdot}05$).

EGG TRAITS

The fertilization rate was unrelated to body size (mixed-model GLM, d.f. = 2 and 69, $P > 0{\cdot}05$). It was slightly, but not significantly, higher among large- (52·3 ± 3·1%; $n = 60$) and random-sized fish (51·5 ± 3·4%; $n = 52$) compared to small fish (42·2 ± 4·0%; $n = 36$). Small females (0·899 ± 0·002 mm) produced significantly larger eggs than random-sized (0·879 ± 0·002 mm) and large females (0·862 ± 0·003) when egg size was measured as egg diameter [Fig. 2(a)]. The trend remained but the difference was no longer significant when egg size was measured as egg yolk diameter: small (0·525 ± 0·002 mm), random-sized (0·523 ± 0·001 mm) and large fish (0·521 ± 0·001 mm) produced eggs with similar yolk sizes [Fig. 2(b)]. The egg size varied significantly between the spawning days in terms of both

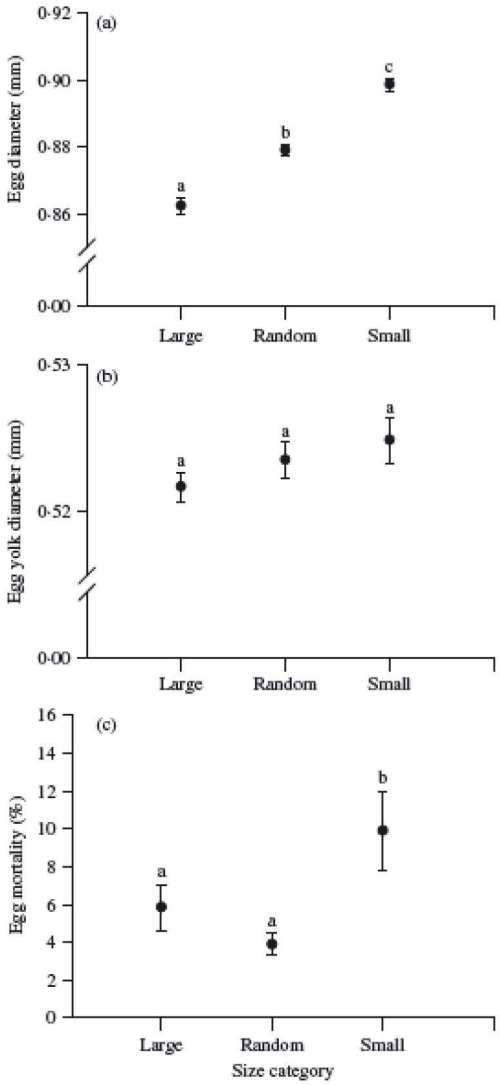

FIG. 2. The effect of *Danio rerio* body size on mean ± S.E. (a) egg diameter (repeated-measures ANOVA, d.f. = 2 and 227, $P < 0{\cdot}001$; large fish: $n = 48$, random-sized fish: $n = 93$ and small fish: $n = 89$), (b) egg yolk diameter (d.f. = 2 and 171, $P > 0{\cdot}05$; large fish: $n = 64$, random-sized fish: $n = 61$ and small fish: $n = 49$) and (c) egg mortality (d.f. = 2 and 24, $P < 0{\cdot}05$; large fish: $n = 10$, random-sized fish: $n = 10$ and small fish: $n = 7$). Different lowercase letters indicate significant differences.

egg diameter (repeated-measures ANOVA, d.f. = 2 and 226, $P < 0{\cdot}01$) and egg yolk diameter (d.f. = 2 and 170, $P < 0{\cdot}001$). There was also a significant interaction between size category and spawning day in egg diameter (d.f. = 4 and 452, $P < 0{\cdot}001$) [Fig. 3(a)] but not in egg yolk size (d.f. = 4 and 340, $P > 0{\cdot}05$). In

neither of the egg size analyses were the C.V. between size classes significantly different.

Eggs from small fish exhibited significantly higher mortality ($9{\cdot}9 \pm 2{\cdot}1\%$) than eggs spawned by large ($5{\cdot}8 \pm 1{\cdot}2\%$) and random-sized fish ($3{\cdot}9 \pm 0{\cdot}6\%$) [Fig. 2(c)]. Spawning day had a significant effect on egg mortality rate (repeated-measures ANOVA, d.f. $= 4$ and 21, $P < 0{\cdot}001$) and also the interaction between size category and spawning day was significant (d.f. $= 8$ and 42, $P < 0{\cdot}05$) [Fig. 3(b)].

LARVAL TRAITS

Larvae from large ($4{\cdot}9 \pm 10{\cdot}1$ days) and random fish ($5{\cdot}2 \pm 0{\cdot}1$ days) hatched significantly earlier than larvae from small fish ($5{\cdot}6 \pm 0{\cdot}1$ days; Fig. 4). L_S-at-hatch did not differ significantly between large ($3{\cdot}10 \pm 0{\cdot}03$ mm) and small fish ($3{\cdot}04 \pm 0{\cdot}03$ mm), while random-sized fish produced significantly larger larvae ($3{\cdot}19 \pm 0{\cdot}03$ mm; repeated-measures ANOVA, d.f. $= 2$ and 55, $P < 0{\cdot}01$). Spawning day did not have a significant effect on larval L_S (d.f. $= 2$ and 55, $P > 0{\cdot}05$) neither did egg size (d.f. $= 1$ and 57, $P > 0{\cdot}05$). The interaction between size category and spawning day, however, was significant in terms of larval L_S-at-hatch (d.f. $= 4$ and 110, $P < 0{\cdot}001$) [Fig. 3(c)]. The larvae of small fish had significantly lower area-at-hatch ($0{\cdot}95 \pm 0{\cdot}02$ mm^2) than the offspring of random-sized ($1{\cdot}04 \pm 0{\cdot}01$ mm^2) and large fish ($1{\cdot}01 \pm 0{\cdot}01$ mm^2) [Fig. 5(a)]. The spawning day had a significant effect on larval area-at-hatch when a potential Petri dish effect was controlled (repeated-measures ANOVA, d.f. $= 2$ and 46, $P < 0{\cdot}05$), but no interaction between size category and spawning day was found (d.f. $= 2$ and 92, $P > 0{\cdot}05$). Egg size did not have a significant effect on larval area-at-hatch either (d.f. $= 1$ and 47, $P > 0{\cdot}05$). The yolk-sac volume of the newly hatched larvae was significantly higher among the offspring of large ($0{\cdot}025 \pm 0{\cdot}001$ mm^3) than random-sized ($0{\cdot}023 \pm 0{\cdot}001$ mm^3) and small fish ($0{\cdot}019 \pm 0{\cdot}001$ mm^3) [Fig. 5(b)]. The sampling day had a significant effect on yolk-sac volume (repeated-measures ANOVA, d.f. $= 2$ and 53, $P < 0{\cdot}01$) but no interaction between the size category and the sampling day was found (repeated-measures ANOVA, d.f. $= 6$ and 106, $P > 0{\cdot}05$). Egg size was not found to have an effect on larval yolk-sac volume (d.f. $= 1$ and 54, $P > 0{\cdot}05$). The effect of the Petri dish was not significant in any of these analyses.

OFFSPRING GROWTH

In general, small differences in offspring growth rate between size categories were observed, but the offspring of small fish exhibited significantly higher growth rate in terms of L_S during 60–90 dpf than random-sized or large fish offspring (Table I). Moreover, random-sized fish offspring grew significantly less in terms of M_W than those of large and small fish during the rearing period of 30–60 dpf, but this difference was no longer evident in the rearing period of 60–90 dpf (Table I). At age 90 dpf, offspring of small fish were significantly larger in terms of L_S than the offspring of large and random-sized fish (Table I). Differences in M_W between small, large and random fish offspring, however, were not significant (Table I). At age 90 dpf, the offspring of small fish had significantly lower K than the offspring of random-sized or large fish (Table I and Fig. 6).

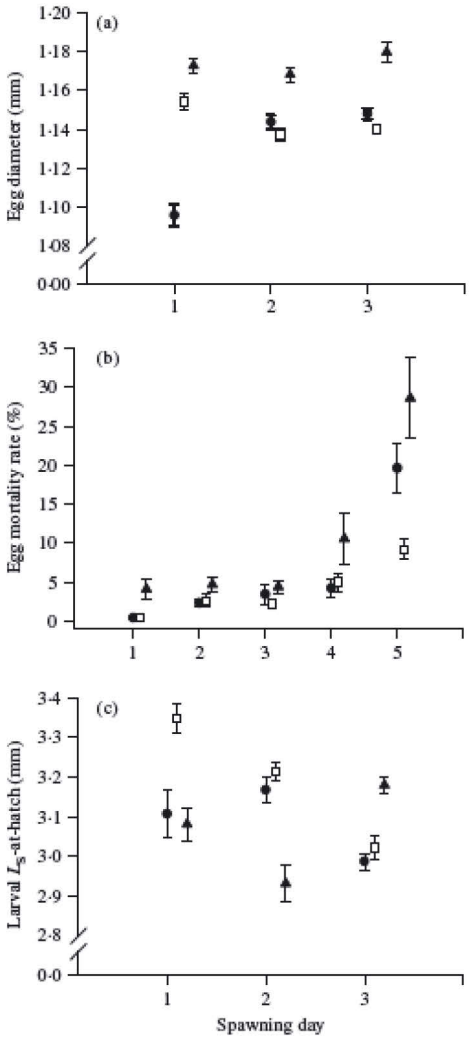

FIG. 3. The effect of spawning day of *Danio rerio* and body size [large (●), random (□) and small (▲)] on mean ± S.E. (a) egg diameter (repeated-measures ANOVA, d.f. = 2 and 226, $P < 0{\cdot}001$; large fish: $n = 48$, random-sized fish: $n = 93$ and small fish: $n = 89$), (b) egg mortality rate (d.f. = 2 and 21, $P < 0{\cdot}05$; large fish: $n = 21$, random-sized fish: $n = 24$ and small fish: $n = 15$) and (c) larval standard length (L_S)-at-hatch (d.f. = 2 and 110, $P < 0{\cdot}05$; large fish: $n = 63$, random-sized fish: $n = 72$ and small fish: $n = 45$).

DISCUSSION

The present study revealed an unambiguous effect of spawner body size on reproductive success in wild *D. rerio* held in the laboratory. Results supported the first hypothesis according to which large females were expected to spawn more

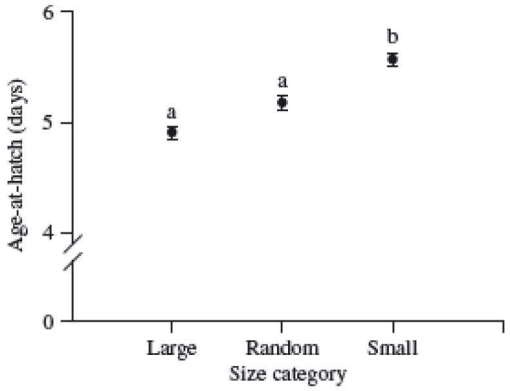

FIG. 4. The effect of *Danio rerio* body size on mean ± S.E. larval age-at-hatch (repeated-measures ANOVA, d.f. = 2 and 238, $P < 0{\cdot}01$). Large fish: $n = 72$, random-sized fish: $n = 55$ and small fish: $n = 114$. Different lowercase letters indicate significant differences.

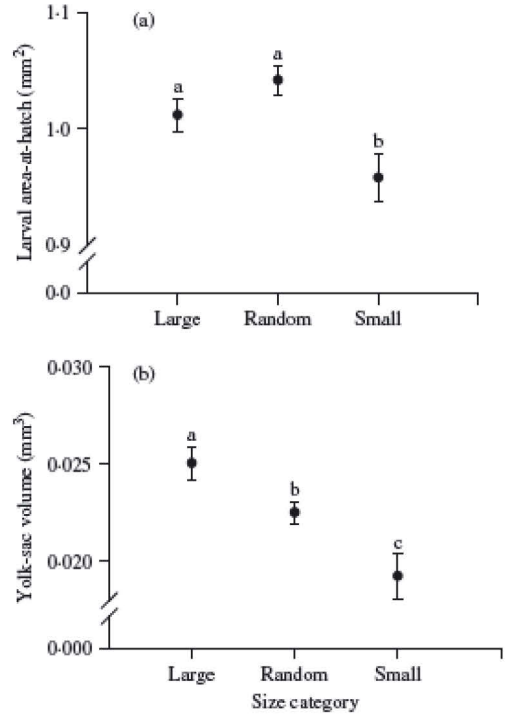

FIG. 5. The effect of *Danio rerio* body size on mean ± S.E. (a) larval area-at-hatch (repeated-measures ANOVA, d.f. = 2 and 47, $P < 0{\cdot}05$; large fish: $n = 14$, random-sized fish: $n = 28$ and small fish: $n = 9$) and (b) larval yolk-sac volume (d.f. = 2 and 54, $P < 0{\cdot}05$; large fish: $n = 15$, random-sized fish: $n = 29$ and small fish: $n = 13$). Different lowercase letters indicate significant differences.

TABLE I. Mean ± S.E. growth rate, standard length (L_S), wet mass (M_W) and condition factor (K) of offspring from three differently sized *Danio rerio* spawners (d.f. = 2) at age 90 days post-fertilization with results of ANOVA tests

Trait	Size group	Mean ± S.E.	F	P
Growth rate	Large	0·24 ± 0·01	0·527	>0·05
30–60 days (mm day^{-1})	Random	0·24 ± 0·01		
	Small	0·22 ± 0·02		
Growth rate	Large	1·83 ± 0·12^{a}	3·383	<0·05
30–60 days (mg day^{-1})	Random	1·50 ± 0·09^{b}		
	Small	2·17 ± 0·26^{a}		
Growth rate	Large	0·14 ± 0·01^{a}	5·544	<0·05
60–90 days (mm day^{-1})	Random	0·13 ± 0·01^{a}		
	Small	0·21 ± 0·01^{b}		
Growth rate	Large	3·39 ± 0·31	0·173	>0·05
60–90 days (mg day^{-1})	Random	3·57 ± 0·30		
	Small	3·36 ± 0·43		
L_S (mm)	Large	19·09 ± 0·31^{a}	6·161	0·01
	Random	18·74 ± 0·34^{a}		
	Small	20·84 ± 0·54^{b}		
M_W (mg)	Large	167·04 ± 7·80	2·629	>0·05
	Random	158·85 ± 9·38		
	Small	186·13 ± 15·73		
K	Large	2·24 ± 0·06^{a}	3·619	<0·05
	Random	2·23 ± 0·05^{a}		
	Small	2·00 ± 0·17^{b}		

Different lowercase superscript letters indicate significant differences ($P < 0·05$).

frequently and produce larger egg clutches than small fish. The general notion of higher fecundity exhibited by large individuals compared to small spawners is in agreement not only with previous studies in *D. rerio* (Spence & Smith, 2006; Paull *et al*., 2008) but also with studies on a number of other fish species (Marteinsdottir

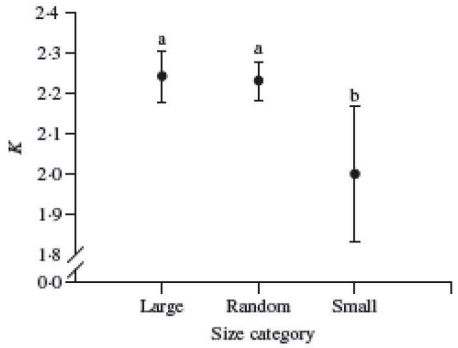

FIG. 6. The effect of *Danio rerio* body size on the offsprings' mean ± S.E. condition factor (K) at age 90 days post-fertilization (two-way ANOVA, d.f. = 2 and 143, $P < 0·05$; large fish: $n = 50$, random-sized fish: $n = 50$ and small fish: $n = 50$). Different lowercase letters indicate significant differences.

& Begg, 2002; Berkeley *et al.*, 2004; Trippel & Neil, 2004). When small *D. rerio* succeeded to spawn, they produced egg numbers per female equal to large females, suggesting that small *D. rerio* may need longer time to accumulate enough energy to produce the same amount of eggs as large females. The average spawning interval was 5 days for large and random-sized fish, which agrees with an earlier study by Hisaoka & Firlit (1962), but it was on average 7 days for small fish with substantial between-day variability. Variance in daily egg production was high among all size groups, which was previously reported from domesticated *D. rerio* strains (Paull *et al.*, 2008). The reasons for the variability in daily egg production can be due to differences in female spawning strategies (Hisaoka & Firlit, 1962; Spence & Smith, 2006) affected by behavioural interactions (Gerlach, 2006).

The second hypothesis, which stressed that large *D. rerio* produce better quality eggs, received mixed empirical support. Contrary to expectations, it was found that small fish produced larger eggs in terms of egg diameter. Small females might compensate for reduced fecundity and spawning frequency by increasing the size of the eggs (Hendry *et al.*, 2001; Murry *et al.*, 2008). Alternatively, small females may produce larger eggs compared to large females due to greater inter-spawning interval (Wootton, 1998), allowing accumulation of energy resources across time before the actual spawning (Kamler, 2005). In the present study, however, changes in egg size and larval L_S-at-hatch did not exhibit a clear decreasing trend over time among large or random-sized spawners (Fig. 3). Thus, the exact mechanism for the greater egg diameter of the eggs spawned by small *D. rerio* remains unclear. Irrespective, data from this study indicate that small females produced eggs with significantly larger perivitelline space (*e.g.* due to swelling; Kamler & Kato, 1983) or thicker chorion (Craik & Harvey, 1984) rather than larger yolk material. Egg yolk is the energy source of a developing embryo and is a better indicator of egg quality than mere egg size (Kamler, 2005). Because the egg yolk did not differ significantly between size categories in the present study, there is no convincing evidence that the larger egg size of small spawners represents a biologically relevant compensatory response.

Despite small fish producing larger eggs, the eggs appeared to be of lower quality, as shown by increased egg mortality rate, compared to large and random-sized spawners. In terms of egg mortality, the quality of eggs appeared to decrease over time in all the size categories [Fig. 3(b)] indicating decreased reproductive quality over the 5 day spawning period. This might be explained by the depletion of energy over time, which may have reduced the amount of energy available for oocyte development (Bagamian *et al.*, 2004). The significantly different egg mortality rate by small and large *D. rerio* spawners suggests size-dependent parental effects on egg quality. Indeed, size-dependent maternal effects have been shown to contribute to embryonic viability *via* egg matter composition rather than egg size (Kamler, 2005) and large females from a number of fish species have been reported to produce higher quality eggs than small females (Zastrow *et al.*, 1989; Brooks *et al.*, 1997; Marteinsdottir & Steinarsson, 1998). Therefore, factors other than egg size alone are likely to contribute to embryo quality and larger egg size of small *D. rerio* is possibly not an indicator of higher quality (see Keckeis *et al.*, 2000). In addition, hormones (Brooks *et al.*, 1997; Nguyen & Zhu, 2009) and sperm quality (Rideout *et al.*, 2004) may influence embryo development and affect egg viability. In *D. rerio*, however, male size and sperm quality do not seem to correlate (Skinner, 2004; Paull *et al.*, 2008), and this is indirectly supported also by the similar fertilization success between small

and large males. Further studies following a factorial breeding design are required to disentangle the relative importance of maternal and paternal effects on egg quality variables in *D. rerio*.

Larvae of large fish hatched earlier and were in a better nutritional condition compared to larvae produced by small fish. Hatching time depends upon the lysis of egg chorion, which is usually thicker in late hatching embryos (Kimmel *et al.*, 1995) and in larger eggs (Craik & Harvey, 1984). In the present study, small fish produced larger eggs, in terms of egg diameter, possibly with thicker chorion than large fish. The strength of the chorion has been reported to vary individually in *D. rerio* (Kimmel *et al.*, 1995), but no correlation with maternal body size has been reported. Hatching time is also known to depend on muscular activity of the embryo, and early hatching larvae have generally higher muscular activity during embryogenesis than late hatching larvae (Kimmel *et al.*, 1995). Thus, early hatching time, which was exhibited in the present study by large fish offspring, is probably an indicator of better larval condition and faster development rate.

Despite egg size being commonly considered to correlate with larval size-at-hatch (Chambers & Leggett, 1996; Pepin *et al.*, 1997; Marteinsdottir & Begg, 2002), such a relationship was not evident in the present data. Large fish offspring had identical L_S-at-hatch but significantly higher area-at-hatch and yolk-sac volume than small fish offspring. This suggests that factors other than egg size may be decisive in embryogenesis defining larval size-at-hatch (Kamler, 2005). In fact, larvae vary in their developmental and yolk absorption rates (Kamler, 2008); thus embryonic growth rate and efficiency at which yolk energy is converted to body tissues during the embryogenesis may result in different hatching time, larval area-at-hatch and yolk-sac volume despite similar egg yolk volumes. Furthermore, differences in yolk energy content, which results not only from the yolk size but also from the caloric value of yolk, can lead to different quality eggs and larvae (Kamler, 2005). These mechanisms may explain the higher area-at-hatch and yolk-sac volume of larvae produced by large fish, suggesting that they were in better condition than offspring of small *D. rerio*.

Larger larvae in terms of body area and yolk-sac volume produced by large fish had a higher K value at age 90 dpf, which supported the third hypothesis of the present study. Contrary to expectations, however, offspring of large fish did not express higher somatic growth rates during early ontogeny compared to larvae produced by small fish. This finding contradicts other studies according to which initial size differences of the offspring from differently sized parents may be maintained for several weeks into the juvenile life stage (Chambers *et al.*, 1988, 1989; Einum & Fleming, 1999; Lindholm *et al.*, 2006). In fact, smaller larvae produced by small *D. rerio* exhibited higher growth rates (in terms of L_S) in a particular juvenile phase (60–90 dpf) than larvae originating from large spawners. This might be indicative of compensatory growth, as offspring of small fish may have compensated the lower energy reserves of the yolk sac by growing relatively fast during the early juvenile phase (Ali *et al.*, 2003).

Population density and skewed sex ratio have been shown to influence *D. rerio* mating behaviour and success through increased aggression (Pritchard, 2001; Spence & Smith, 2005) potentially confounding the effect of body size on reproductive variables. The present study controlled for these effects by keeping the density and sex ratio similar in all trials for all size categories. The establishment of dominance hierarchies might suppress the resource utilization of subdominant fish, and it may

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be argued that small fish might have been undernourished; hence, nutritional state rather than body size explains the study results. This is, however, unlikely because K of small, random and large fish did not differ after the holding period. K has been shown to be an unreliable measure of the fish energy stores in the wild because it may fluctuate seasonally stemming from reproductive cycles and varying food availability (Le Cren, 1951; Fechhelm *et al.*, 1995). In *D. rerio*, however, K has been shown to reflect differences in feeding regimes and thus in feeding opportunities and nutritional state (S. Uusi-Heikkilä, A. Kuparinen, C. Wolter, T. Meinelt, A. C. O'Toole & R. Arlinghaus, unpubl. data). The potential existence of dominance hierarchies during the holding phase should therefore not have affected the general size effect on reproductive traits reported here unless new dominance hierarchies were established during the reproductive trials influencing the reproductive output differently between size categories.

To conclude, a spawning stock composed of large *D. rerio* had higher reproductive output compared to small individuals. Furthermore, eggs produced by large females appeared to be of higher quality by exhibiting significantly lower mortality rates than eggs produced by small females. It can thus be concluded that there are size-dependent parental effects in laboratory-held wild *D. rerio*, which are reflected in egg, larval and offspring quality but not necessarily in juvenile growth rate. Further studies are needed to investigate the mechanisms behind the differences in egg and larval quality between large- and small-sized parents. In particular, there is a need to disentangle the relative importance of maternal and paternal effects on reproductive output and egg and larval traits in *D. rerio*. Such studies are needed to inform fisheries models about the impact of size-selective mortality on recruitment dynamics and also to help guide laboratory protocols for studies on *D. rerio*, which depend on unequivocal assessment of reproductive variables.

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Paper V

V

A behavioral perspective on fishing-induced evolution

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A behavioral perspective on fishing-induced evolution

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The potential for excessive and/or selective fishing to act as an evolutionary force has been emphasized recently. However, most studies have focused on evolution of life-history traits in response to size-selective harvesting. Here we draw attention to fishing-induced evolution of behavioral and underlying physiological traits. We contend that fishing-induced selection directly acting on behavioral rather than on life-history traits *per se* can be expected in all fisheries that operate with passive gears such as trapping, angling and gill-netting. Recent artificial selection experiments in the nest-guarding largemouth bass *Micropterus salmoides* suggest that fishing-induced evolution of behavioral traits that reduce exposure to fishing gear might be maladaptive, potentially reducing natural recruitment. To improve understanding and management of fisheries-induced evolution, we encourage greater application of methods from behavioral ecology, physiological ecology and behavioral genetics.

The potential for fishing-induced evolution (FIE) has been discussed recently [1,2]. Most studies reviewed in Ref. [1] have focused on life-history traits that directly or indirectly determine body size. Under the common scenario of size-selective harvesting, large fish face a fitness disadvantage that might cause rapid evolution toward earlier maturation at smaller sizes, higher reproductive investment and lower intrinsic growth capacity and, collectively, smaller size-at-age [2]. Such evolution can degrade fisheries yield and other ecological services within decades [2].

Many studies on FIE, however, fall short in addressing the selection pathways that drive the observed life-history changes. For example, evolution of small body size can result from direct selection for decreased intrinsic growth capacity or be a consequence of selection on correlated life-history or behavioral traits [3]. Indeed, in some passively operated fishing gears (e.g. trapping, angling, gill-netting), behavioral traits rather than body size *per se* determine a fish's vulnerability to capture, and thus its survival and fitness (Figure 1) [3]. In these situations, direct selection on behavior can drive evolutionary changes in correlated life-history traits such as growth rate [3] because the more active, bold and vulnerable individuals tend to also grow faster [4,5]. Despite the important role of behavior in influencing catchability in various fisheries [3,6–8], the behavioral dimension of FIE has largely been neglected.

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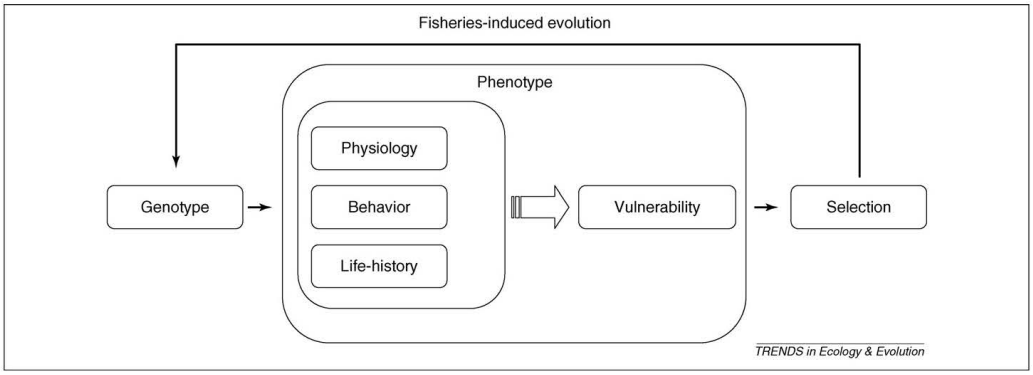

Figure 1. Mechanistic pathway of fishing-induced evolution by selection on fishing vulnerability. In this scheme, vulnerability to capture is considered a heritable trait as part of the fish's phenotype. Vulnerability to capture comprises a bundle of physiological, behavioral and life-history traits that jointly determine vulnerability to capture. In passive fisheries, vulnerability to capture is largely determined by specific behavioral patterns rather than by body size-related life-history traits *per se*. Due to genetic correlations between behavioral, physiological and life-history traits, fisheries-induced selection on behavioral traits might alter physiologies and life histories, but behavior might also change in response to selection on correlated life-history or other traits.

Evolutionary responses to fisheries-induced selection depend on the selection differential and the heritability of the trait [1]. Large selection pressures on behavioral traits can be expected when specific behavioral patterns increase the encounter probability with the fishing gear, thus influencing survival and fitness. For example, vulnerability to capture by gill-nets not only depends on body size and shape but is also strongly related to an individual's activity level [3,7]. Similarly, in recreational angling, vulnerability to capture can be size related, but most importantly depends on a fish's decision to attack and/or ingest baited hooks [7–9]. In this context, bold and aggressive personalities, individuals with lower cognitive abilities and those with higher metabolism and growth capacity often take more risks and hide less in structured habitat, rendering these fish more vulnerable to capture [3,7,8]. Thus, behavior-driven vulnerability to fishing might constitute an underappreciated mechanism for selection on growth rate [3] or other life-history traits [5]. Alternatively, due to genetic correlations between physiological, behavioral and life-history traits (Figure 1), evolution of behavioral traits might be an indirect consequence of selection on body size under strongly size-selective harvesting. Collectively, if exploitation directly or indirectly induces a large selection differential on particular heritable behavioral traits, evolving fish stocks will not only become less abundant and smaller [2] but also harder to catch [3,6–9], which diminishes the quality of the fishery.

Selection responses of behavioral traits to fishing can be rapid because heritabilities of behavioral traits are often larger than those of life-history traits [10,11]. Indeed, in largemouth bass (*Micropterus salmoides*), artificial selection for vulnerability to recreational angling induced evolutionary changes in various physiological and behavioral traits after only four generations [8]. Vulnerable individuals had higher metabolic rates and resting cardiac activity, and provided more intense parental care than invulnerable fish of the same body size [8]. Vulnerability to capture therefore was primarily determined by physiological and behavioral traits rather than by body size. This suggests that selective harvest of highly vulnerable largemouth bass could impact the population in the long term by altering parental care activity and level of aggression [8]. Moreover, in nest-guarding species, FIE is conceivable even in the absence of fishing mortality, for example when recreational anglers practice catch-and-release during the reproductive period [8]. In these situations, the fitness of more aggressive and vulnerable individuals is reduced when they are temporarily removed from their nests, leaving the brood susceptible to rapid egg predation [12]. Over time, this might favor more wary and less vulnerable genotypes that happen to also be inferior nest guarders.

The potential for evolution of behavioral and physiological traits and its consequences for life history, demography and fishing quality constitutes a fascinating, yet largely overlooked research area within the emerging field of FIE. To improve understanding and management of FIE, we encourage collaboration between fishery scientists and evolutionary ecologists (*sensu* [1]), and greater application of methods from behavioral ecology, physiological ecology and behavioral genetics.

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